

# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/040709

International filing date: 03 December 2004 (03.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US  
Number: 60/527,082  
Filing date: 03 December 2003 (03.12.2003)

Date of receipt at the International Bureau: 17 January 2005 (17.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1270178



THE UNITED STATES OF AMERICA

TO ALL WHOM THESE PRESENTS SHALL COME

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

*January 06, 2005*

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM  
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK  
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT  
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A  
FILING DATE.

APPLICATION NUMBER: 60/527,082

FILING DATE: *December 03, 2003*

RELATED PCT APPLICATION NUMBER: PCT/US04/40709



Certified By



Jon W Dudas

Under Secretary  
of Commerce for Intellectual Property  
and Acting Director of the  
United States Patent and Trademark Office

120303  
16638 U.S.PTO

PTO/SB/16 (08-03)

## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 317114892US

## INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Shawn	DeFrees	North Wales, PA
Robert J.	Bayer	San Diego, CA

 Additional inventors are being named on the separately numbered sheets attached hereto

## TITLE OF THE INVENTION (500 characters max)

GLYCOPEGYLATED HUMAN FOLLICLE STIMULATING HORMONE

Direct all correspondence to: CORRESPONDENCE ADDRESS

 Customer Number

20350

OR

Firm or  
Individual Name

Address

City

State

ZIP

Country

Telephone

Fax

15535 U.S.PTO  
60/527082

120303

## ENCLOSED APPLICATION PARTS (check all that apply)

 Specification Number of Pages

77

 CD(s), Number Drawing(s) Number of Sheets

6

 Other (specify) Application Data Sheet. See 37 CFR 1.76

## METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

 Applicant claims small entity status. See 37 CFR 1.27. A check or money order is enclosed to cover the filing fees.FILING FEE  
AMOUNT (\$) The Director is hereby authorized to charge filing  
fees or credit any overpayment to Deposit Account Number: 20-1430

160

 Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

 No. Yes, the name of the U.S. Government agency and the Government contract number are: .

[Page 1 of 1]

Date 12/03/03

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

TELEPHONE

REGISTRATION NO. 42,837

(if appropriate)

Docket Number: 019957-019300US

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

60093863 v1

**PROVISIONAL PATENT APPLICATION**

**GLYCOPEGYLATED FOLLICLE STIMULATING HORMONE**

Inventor(s): Shawn DeFrees, a citizen of The United States, residing at  
126 Filly Drive  
North Wales, PA 19454

Robert J. Bayer, a citizen of The United States, residing at  
6105 Dirac Street  
San Diego, CA 92122

Assignee: Neose Technologies, Inc.  
102 Witmer Road  
Horsham, PA 19044

Entity: Small business concern

Jeffry S. Mann, Ph.D.  
Reg. No. 42,837

---

TOWNSEND  
*and*  
TOWNSEND  
*and*  
CREW LLP

---

Two Embarcadero Center  
Eighth Floor  
San Francisco  
California 94111-3834  
Tel 415 576-0200  
Fax 415 576-0300

---

**AS FILED IN THE U.S. PTO ON DECEMBER 3, 2003**

## GLYCOPEGYLATED FOLLICLE STIMULATING HORMONE

### BACKGROUND OF THE INVENTION

The administration of glycosylated and non-glycosylated peptides for engendering a particular physiological response is well known in the medicinal arts. Among the best known peptides utilized for this purpose is insulin, which is used to treat diabetes. Enzymes have also been used for their therapeutic benefits. A principal factor, which has limited the use of therapeutic peptides is the immunogenic nature of most peptides. In a patient, an immunogenic response to an administered peptide can neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of therapeutic glycopeptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the problems have been investigated. To provide soluble peptide therapeutics, synthetic polymers have been attached to the peptide backbone.

Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to peptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) concerns non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide and at least 15% of the physiological activity is maintained. In addition, the clearance time in circulation is prolonged due to the increased size of the PEG-conjugate of the polypeptides in question.

WO 93/15189 (Veronese *et al.*) concerns a method to maintain the activity of polyethylene glycol-modified proteolytic enzymes by linking the proteolytic enzyme to a macromolecularized inhibitor. The conjugates are intended for medical applications.

The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently bound to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of  $\alpha$ -1 proteinase inhibitor with a polymer such as PEG or methoxypoly(ethyleneglycol) ("MPEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977) discloses the covalent attachment of MPEG to an amine group of bovine serum

albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon- $\beta$ , interleukin-2 and immunotoxins. 5 EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

Another mode of attaching PEG to peptides is through the non-specific 10 oxidation of glycosyl residues on a glycopeptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example M'Timkulu (WO 94/05332) discloses the use of an amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG.

In each of the methods described above, poly(ethyleneglycol) is added in a 15 random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide.

20 Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (e.g., sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as 25 exoglycosidases (e.g.,  $\beta$ -mannosidase,  $\beta$ -glucosidase), and endoglycosidases (e.g., Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, see, Crout *et al.*, *Curr. Opin. Chem. Biol.* 2: 98-111 (1998).

Glycosyltransferases modify the oligosaccharide structures on glycopeptides. 30 Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of

glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example,  $\beta$ -1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of 5 carbohydrates (see, e.g., Wong *et al.*, *J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of  $\alpha$ -sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneurameric acid to the 3-OH or 6-OH of galactose (see, e.g., Kevin *et al.*, *Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific 10 hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al.*, *J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use see, Koeller *et* 15 *al.*, *Nature Biotechnology* **18**: 835-841 (2000). See also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. 20 The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the notoriously difficult  $\beta$ -mannoside linkage, which was formed by the action of  $\beta$ -mannosidase (Singh *et* 25 *al.*, *Chem. Commun.* 993-994 (1996)).

In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant 30 glycosidases are used to prepare oligosaccharides using an  $\alpha$ -glycosyl fluoride donor and a glycoside acceptor molecule (Withers *et al.*, U.S. Patent No. 5,716,812). Although the mutant glycosidases are useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated

or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of 5 endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*- $\beta$ -N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al.*, *Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al.*, *Carbohydr. Res.* **292**: 61-70 (1996)).

In addition to their use in preparing carbohydrates, the enzymes discussed 10 above are applied to the synthesis of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al.*, *J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on 15 the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of  $\beta$ -1,4-galactosyltransferase,  $\alpha$ -2,3-sialyltransferase and  $\alpha$ -1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

Methods combining both chemical and enzymatic synthetic elements are also 20 known. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglycosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin glycopeptide. The saccharide portion was added to the peptide by treating 25 it with an *endo*- $\beta$ -N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

The use of glycosyltransferases to modify peptide structure with reporter 30 groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and gangliosides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation

of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays 5 for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested.

10 Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al.*, *J. Biol. Chem.* 271: 27213 (1996)).

15 Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing 20 modifying group. For example, Casares *et al.* (*Nature Biotech.* 19: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

25 Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (*Science* 276: 1125 (1997)) have prepared N-levulinoyl mannosamine (“ManLev”), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. See, also Saxon *et al.*, *Science* 287: 2007 (2000); Hang *et al.*, *J. Am. Chem. Soc.* 123: 1242 (2001); Yarema *et al.*, *J. Biol. Chem.* 273: 31168 (1998); and Charter *et al.*, *Glycobiology* 10: 1049 (2000).

30 The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Moreover, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

Despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for an industrially practical method for the modification of glycosylated and non-glycosylated peptides with modifying groups such as PEG moieties, therapeutic moieties, biomolecules and the like. Of particular interest are methods in which 5 the modified peptide has improved properties, which enhance its use as a therapeutic or diagnostic agent.

Follicle Stimulating Hormone (FSH) is an exemplary therapeutic peptide that is presently underutilized due to its less than ideal pharmacological properties. FSH is a vital component of the regulatory cascade governing development of human oocytes. Primary 10 oocytes in newborns are arrested in the prophase stage of Meiosis I and are surrounded by a 1-2 cell thick layer of follicle cells constituting a structure termed the primordial follicle. In concert with other factors, stimulation of the primordial follicle with FSH initiates its progression to the more complex structures designated the developing and antral follicles (Ueno, et al., *Proc. Natl. Acad. Sci. USA* **84**: 8282-8286 (1987); Robertson et al., *Biochem. 15 Biophys. Res. Comm.* **149**: 744-749 (1987)).

Follicle stimulating hormone, either extracted from urine or produced by recombinant DNA technology, is a parenterally-administered protein product used by specialists for ovulation induction (OI) and for controlled ovarian hyperstimulation (COH). Whereas OI is directed at achieving a single follicle to ovulate, COH is directed at harvesting 20 multiple oocytes for use in various in vitro assisted reproductive technologies (e.g., for in vitro fertilization). Clinical use of preparations containing FSH began in the 1960's.

Follicle stimulating hormone (FSH) is a pituitary-derived heterodimeric glycoprotein hormone that shares structural similarities with luteinizing hormone (LH) and thyroid stimulating hormone (TSH), both of which are also produced in the pituitary gland, 25 and chorionic gonadotropin (CG), which is produced in the placenta. The hormones are relatively large (28-38 kilodaltons) and are composed of a common  $\alpha$  subunit non-covalently bound to a distinct  $\beta$  subunit that confers receptor binding specificity.

The cellular receptors for these hormones are known to be members of the G protein-coupled class of membrane-bound receptors, which, when activated, stimulate an 30 increase in the activity of adenylyl cyclase. This results in an increase in the level of the intracellular second messenger adenosine 3', 5'-monophosphate (cAMP), which in turn causes increased steroid synthesis and secretion. Hydropathicity plots of the amino acid sequences of these receptors reveal three general domains: (1) a hydrophilic amino-terminal region,

considered to be the amino-terminal extracellular domain, (2) seven hydrophobic segments of membrane-spanning length, considered to be the transmembrane domain, and (3) a carboxy-terminal region that contains potential phosphorylation sites (serine, threonine, and tyrosine residues), considered to be the carboxy-terminal intracellular or cytoplasmic domain. The 5 glycoprotein hormone receptor family is distinguished from other G protein-coupled receptors, such as the  $\beta$ -2-adrenergic, rhodopsin, and substance K receptors, by the large size of the hydrophilic amino-terminal domain, which is involved in hormone binding.

The FSH receptor is expressed on testicular Sertoli cells and ovarian granulosa cells. While there has been a recognized need for providing essentially pure human FSH 10 receptor, purification of naturally derived preparations is not practical and would likely be insufficient to permit determination of the amino acid sequence. Recently, the cDNA encoding the rat FSH receptor was cloned, the amino acid sequence was deduced, and the peptide was expressed in mammalian cells (Sprengel, *Mol. Endocrinol.* 4: 525 (1990)). Another group, attempting to clone the TSH receptor, apparently also cloned and identified a 15 portion of the transmembrane region of the human FSH receptor (Parmentier, *Science* 246: 1620 (1989)).

At present, use of FSH is limited by its high cost, lack of oral dosing, and need 20 of extensive monitoring by specialist physicians. Hence, identification of a non-peptidic small molecule substitute for FSH that could potentially be developed for oral administration is desirable.

In response to the need for improved therapeutic FSH, the present invention provides a glycopegylated FSH that is therapeutically active and which has pharmacokinetic parameters and properties that are improved relative to an identical, or closely analogous, FSH peptide that is not glycopegylated.

## 25 BRIEF SUMMARY OF THE INVENTION

It has now been discovered that the controlled modification of Follicle Stimulating Hormone (FSH) with one or more poly(ethylene glycol) moieties affords a novel 30 FSH derivative with pharmacokinetic properties that are improved relative to the corresponding native (un-pegylated) FSH (FIG. 3). Moreover, the bioavailability of the glycopegylated FSH is equal to or better than that of the native FSH (FIG. 4).

In an exemplary embodiment, “glycopegylated” FSH molecules of the invention are produced by the enzyme mediated formation of a conjugate between a glycosylated or non-glycosylated FSH peptide and an enzymatically transferable saccharyl

moiety that includes a poly(ethylene glycol) moiety within its structure. The PEG moiety is attached to the saccharyl moiety directly (i.e., through a single group formed by the reaction of two reactive groups) or through a linker moiety, e.g., substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, etc. An exemplary transferable PEG-saccharyl

5 structure is set forth in **FIG. 5.**

Thus, in a first aspect, the present invention provides a conjugate between a PEG moiety, e.g., PEG and a peptide that has an *in vivo* activity similar or otherwise analogous to art-recognized FSH. In the conjugate of the invention, the PEG moiety is covalently attached to the peptide via an intact glycosyl linking group. Exemplary intact 10 glycosyl linking groups include sialic acid moieties that are derivatized with PEG.

The pharmacokinetic properties of the compounds of the invention are readily varied by altering the structure, number or position of the glycosylation site(s) of the peptide. Thus, it is within the purview of the present application to add one or more mutation that inserts an O- or N-linked glycosylation site into the FSH peptide that is not present in the 15 wild type. Antibodies to these mutants and their glycosylated final products and intermediates are also within the scope of the present invention.

In a second aspect, the invention provides a FSH conjugate having a population of PEG moiety moieties, e.g., PEG, covalently bound thereto through an intact glycosyl linking group. In the conjugate of the invention, essentially each member of the 20 population is bound via the glycosyl linking group to a glycosyl residue of the peptide, and each glycosyl residue has the same structure.

In a third aspect, the present invention provides a FSHconjugate having a population of PEG moiety moieties, e.g., PEG, covalently bound thereto through an intact glycosyl linking group. In the conjugate of the invention, essentially each member of the 25 population is bound to an amino acid residue of the peptide, and each of the amino acid residues to which the polymer is bound has the same structure. For example, if one peptide includes a Ser linked glycosyl residues, at least about 70%, 80%, 90%, 95%, 97%, 99%, 99.2%, 99.4%, 99.6%, or more preferably 99.8% of the peptides in the population will have the same glycosyl residue covalently bound to the same Ser residue. The discussion above is 30 equally relevant for other O-glycosylation and N-glycosylation sites.

In a fourth aspect, the present invention provides a method of forming a covalent conjugate between a PEG moiety and a glycosylated or non-glycosylated FSH peptide. The polymer is conjugated to the peptide via an intact glycosyl linking group, which is interposed between and covalently linked to both the peptide and the polymer. The method

includes contacting the peptide with a mixture containing a sugar covalently modified with the polymer. An exemplary sugar has a structure such as that shown in **FIG. 5**. The mixture also includes a glycosyltransferase for which the modified sugar is a substrate. The transferase is selected to add saccharyl donors to amino acid or glycosyl residues on the peptide. The reaction is conducted under conditions sufficient to form the conjugate.

5 In a further aspect, the present invention provides a composition for forming a conjugate between a FSH peptide and a PEG-modified sugar. The composition includes a mixture of a modified sugar, a glycosyl transferase, and a FSH peptide acceptor substrate. The modified sugar has a PEG moiety covalently attached thereto, either directly or through a  
10 non-glycosyl linker.

15 Also provided is a pharmaceutical composition. The composition includes a pharmaceutically acceptable carrier and a covalent conjugate between a non-naturally-occurring, PEG moiety and a glycosylated or non-glycosylated FSH peptide. The polymer is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer. The polymer is attached to the glycosyl directly or through a non-glycosyl linker arm, e.g. substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, etc.

20 Other objects and advantages of the invention will be apparent to those of skill in the art from the detailed description that follows.

20

## DESCRIPTION OF THE DRAWINGS

**FIG. 1** is the structure of FSH, showing the presence and location of the glycosyl moieties at Asn 7, Asn 24, Asn 52 and Asn 78.

25 **FIG. 2 (A)** is a scheme showing an exemplary embodiment of the invention in which a carbohydrate residue on a FSH peptide is remodeled by removing a sialic acid moiety from each of two saccharyl residues prior to adding a saccharyl moiety derivatized with PEG: (B) is A SDS PAGE gel showing the relative molecular sizes of native FSH, asialo-FSH and FSH glycopegylated with 1 Kd PEG.

30 **FIG. 3** is a plot comparing the in vivo residence lifetimes of native FSH and glycopegylated FSH.

**FIG. 4** is a plot comparing the bioactivities of native FSH and glycopegylated FSH at selected dosages.

**FIG. 5** is a synthetic scheme for producing an exemplary PEG-glycosyl linking group precursor (modified sugar) of us in preparing the conjugates of the invention.

**FIG. 6** is the amino acid sequence of FSH.

## **DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS**

### **Abbreviations**

5 PEG, poly(ethyleneglycol); PPG, poly(propyleneglycol); Ara, arabinosyl; Fru, fructosyl; Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Xyl, xylosyl; and NeuAc, sialyl (N-acetylneuraminy); M6P, mannose-6-phosphate.

10 **Definitions**

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry 15 and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is 20 incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

25 All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (*i.e.*, Gal), followed by the configuration of the glycosidic bond ( $\alpha$  or  $\beta$ ), the ring bond (1 or 2), the ring position of the reducing saccharide involved in the bond (2, 3, 4, 6 or 8), and then the name or abbreviation of the reducing 30 saccharide (*i.e.*, GlcNAc). Each saccharide is preferably a pyranose. For a review of standard glycobiology nomenclature see, *Essentials of Glycobiology* Varki *et al.* eds. CSHL Press (1999).

Oligosaccharides are considered to have a reducing end and a non-reducing end, whether or not the saccharide at the reducing end is in fact a reducing sugar. In

accordance with accepted nomenclature, oligosaccharides are depicted herein with the non-reducing end on the left and the reducing end on the right.

The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* **265**: 10 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C<sub>1</sub>-C<sub>6</sub> acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* **2**: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation 15 procedure is disclosed in international application WO 92/16640, published October 1, 1992.

"Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example,  $\beta$ -alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L - isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and 20 unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see*, Spatola, A. F., in *CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

The term "peptide conjugate," refers to species of the invention in which a 30 peptide is conjugated with a modified sugar as set forth herein.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by

the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline,  $\gamma$ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an  $\alpha$  carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, 5 norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

10 As used herein, the term “modified sugar,” refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (*e.g.*, glycosyl halides, glycosyl mesylates) and 15 sugars that are neither activated nor nucleotides. The “modified sugar” is covalently functionalized with a “modifying group.” Useful modifying groups include, but are not limited to, PEG moieties, therapeutic moieties, diagnostic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is selected such that it 20 does not prevent the “modified sugar” from being added enzymatically to a peptide.

The term “water-soluble” refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary PEG moieties include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences or be composed of a 25 single amino acid, *e.g.* poly(lysine). Similarly, saccharides can be of mixed sequence or composed of a single saccharide subunit, *e.g.* dextran, amylose, chitosan, and poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol). Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid)

30 The term, “glycosyl linking group,” as used herein refers to a glycosyl residue to which an agent (*e.g.*, PEG moiety, therapeutic moiety, biomolecule) is covalently attached. In the methods of the invention, the “glycosyl linking group” becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A “glycosyl linking group” is generally derived from a “modified sugar” by the enzymatic attachment of the “modified sugar” to an amino acid

and/or glycosyl residue of the peptide. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the individual saccharide monomer that links the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring  
5 oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

The term "targeting moiety," as used herein, refers to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or  
10 conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins,  $\beta$ -glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

15 As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers  
20 may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known  
25 conventional methods.

As used herein, "administering," means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, or the implantation of a slow-release device e.g., a mini-osmotic pump, to the subject. Adminsitration is by any route including parenteral, and  
30 transmucosal (e.g., oral, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, e.g., induce apoptosis, administration may be directly to the tumor and/or into tissues

surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

The term “isolated” refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term “isolated” refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. “Isolated” and “pure” are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or 10 more than about 90%.

When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is 15 about 92%, about 94%, about 96%, about 98% or about 100% purity.

Purity is determined by any art-recognized method of analysis (e.g., band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

“Essentially each member of the population,” as used herein, describes a 20 characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. “Essentially each member of the population” speaks to the “homogeneity” of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and 25 more preferably at least about 95% homogenous.

“Homogeneity,” refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the 30 peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or 5 about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, e.g., liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDI-TOF), capillary electrophoresis, and the like.

“Substantially uniform glycoform” or a “substantially uniform glycosylation 10 pattern,” when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (e.g., fucosyltransferase). For example, in the case of a  $\alpha$ 1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Gal $\beta$ 1,4-GlcNAc-R and sialylated 15 analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (e.g., fucosylated Gal $\beta$ 1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

The term “substantially” in the above definitions of “substantially uniform” 20 generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

Where substituent groups are specified by their conventional chemical 25 formulae, written from left to right, they equally encompass the chemically identical substituents, which would result from writing the structure from right to left, e.g., -CH<sub>2</sub>O- is intended to also recite -OCH<sub>2</sub>-.

The term “alkyl,” by itself or as part of another substituent means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and 30 multivalent radicals, having the number of carbon atoms designated (i.e. C<sub>1</sub>-C<sub>10</sub> means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example,

n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and 5 isomers. The term “alkyl,” unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as “heteroalkyl.” Alkyl groups that are limited to hydrocarbon groups are termed “homoalkyl”.

The term “alkylene” by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by  $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$ , and 10 further includes those groups described below as “heteroalkylene.” Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A “lower alkyl” or “lower alkylene” is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The terms “alkoxy,” “alkylamino” and “alkylthio” (or thioalkoxy) are used in 15 their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

The term “heteroalkyl,” by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one 20 heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to,  $-\text{CH}_2\text{-CH}_2\text{-O-CH}_3$ ,  $-\text{CH}_2\text{-CH}_2\text{-NH-CH}_3$ ,  $-\text{CH}_2\text{-CH}_2\text{-N}(\text{CH}_3)\text{-CH}_3$ ,  $-\text{CH}_2\text{-S-CH}_2\text{-CH}_3$ ,  $-\text{CH}_2\text{-CH}_2\text{-S(O)-CH}_3$ ,  $-\text{CH}_2\text{-CH}_2\text{-S(O)}_2\text{-CH}_3$ ,  $-\text{CH=CH-O-CH}_3$ ,  $-\text{Si}(\text{CH}_3)_3$ ,  $-\text{CH}_2\text{-CH=N-OCH}_3$ , and  $-\text{CH=CH-N}(\text{CH}_3)\text{-CH}_3$ . Up to 25 two heteroatoms may be consecutive, such as, for example,  $-\text{CH}_2\text{-NH-OCH}_3$  and  $-\text{CH}_2\text{-O-Si}(\text{CH}_3)_3$ . Similarly, the term “heteroalkylene” by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by,  $-\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2-$  and  $-\text{CH}_2\text{-S-CH}_2\text{-CH}_2\text{-NH-CH}_2-$ . For heteroalkylene groups, heteroatoms 30 can also occupy either or both of the chain termini (e.g., alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the

formula of the linking group is written. For example, the formula  $-C(O)_2R'$  represents both  $-C(O)_2R'$  and  $-R'C(O)_2$ .

The terms “cycloalkyl” and “heterocycloalkyl”, by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of “alkyl” and “heteroalkyl”, respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

The terms “halo” or “halogen,” by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as “haloalkyl,” are meant to include monohaloalkyl and polyhaloalkyl. For example, the term “halo( $C_1-C_4$ )alkyl” is meant to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term “aryl” means, unless otherwise stated, a polyunsaturated, aromatic, substituent that can be a single ring or multiple rings (preferably from 1 to 3 rings), which are fused together or linked covalently. The term “heteroaryl” refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, 25 pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxaliny, 5-quinoxaliny, 3-quinolyl, tetrazolyl, benzo[b]furanyl, benzo[b]thienyl, 2,3-dihydrobenzo[1,4]dioxin-6-yl, 30 benzo[1,3]dioxol-5-yl and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

For brevity, the term “aryl” when used in combination with other terms (e.g., aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above.

Thus, the term “arylalkyl” is meant to include those radicals in which an aryl group is attached to an alkyl group (e.g., benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (e.g., a methylene group) has been replaced by, for example, an oxygen atom (e.g., phenoxyethyl, 2-pyridyloxymethyl, 3-(1-naphthoxy)propyl, and the like).

Each of the above terms (e.g., “alkyl,” “heteroalkyl,” “aryl” and “heteroaryl”) is meant to include both substituted and unsubstituted forms of the indicated radical.

Preferred substituents for each type of radical are provided below.

Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generically referred to as “alkyl group substituents,” and they can be one or more of a variety of groups selected from, but not limited to: -OR’, =O, =NR’, =N-OR’, -NR’R”, -SR’, -halogen, -SiR’R”R”, -OC(O)R’, -C(O)R’, -CO<sub>2</sub>R’, -CONR’R”, -OC(O)NR’R”, -NR”C(O)R’, -NR’-C(O)NR”R”, -NR”C(O)R”, -NR-C(NR’R”R”)=NR””, -NR-C(NR’R”)=NR””, -S(O)R’, -S(O)<sub>2</sub>R’, -S(O)<sub>2</sub>NR’R”, -NRSO<sub>2</sub>R’, -CN and -NO<sub>2</sub> in a number ranging from zero to (2m’+1), where m’ is the total number of carbon atoms in such radical. R’, R”, R”” and R”” each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R’, R”, R”” and R”” groups when more than one of these groups is present. When R’ and R” are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR’R” is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term “alkyl” is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., -CF<sub>3</sub> and -CH<sub>2</sub>CF<sub>3</sub>) and acyl (e.g., -C(O)CH<sub>3</sub>, -C(O)CF<sub>3</sub>, -C(O)CH<sub>2</sub>OCH<sub>3</sub>, and the like).

Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are generically referred to as “aryl group substituents.” The substituents are selected from, for example: halogen, -OR’, =O, =NR’, =N-OR’, -NR’R”, -SR’, -halogen, -SiR’R”R”, -OC(O)R’, -C(O)R’, -CO<sub>2</sub>R’, -CONR’R”, -OC(O)NR’R”, -NR”C(O)R’, -NR’-C(O)NR”R”, -NR”C(O)R”, -NR-C(NR’R”R”)=NR””, -NR-C(NR’R”)=NR””, -S(O)R’, -S(O)<sub>2</sub>R’, -S(O)<sub>2</sub>NR’R”, -NRSO<sub>2</sub>R’, -CN and -NO<sub>2</sub>, -R’, -

N<sub>3</sub>, -CH(Ph)<sub>2</sub>, fluoro(C<sub>1</sub>-C<sub>4</sub>)alkoxy, and fluoro(C<sub>1</sub>-C<sub>4</sub>)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R'', R''' and R'''' are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. In the schemes that follow, the symbol X represents "R" as described above.

Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may 10 optionally be replaced with a substituent of the formula -T-C(O)-(CRR')<sub>q</sub>-U-, wherein T and U are independently -NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may 15 optionally be replaced with a substituent of the formula -A-(CH<sub>2</sub>)<sub>r</sub>-B-, wherein A and B are independently -CRR'-, -O-, -NR-, -S-, -S(O)-, -S(O)<sub>2</sub>-, -S(O)<sub>2</sub>NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally 20 be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula - (CRR')<sub>s</sub>-X-(CR''R''')<sub>d</sub>-, where s and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -S(O)<sub>2</sub>-, or -S(O)<sub>2</sub>NR'-. The substituents R, R', R'' and R''' are 25 preferably independently selected from hydrogen or substituted or unsubstituted (C<sub>1</sub>-C<sub>6</sub>)alkyl.

As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

Follicle Stimulating Hormone (FSH) is any wild type or mutated peptide, recombinant, or native that has an activity that mimics that of native FSH. The term also 25 generally encompasses non-peptide FSH mimetics.

## Introduction

The present invention provides a conjugate of follicle stimulating hormone (FSH). FSH is a glycoprotein hormone. See, for example, Saneyoshi, *et al.*, *Biol. Reprod.*, 30 65:1686-1690 (2001); Hakola, *et al.*, *J. Endocrinol.*, 158:441-448 (1998); Stanton, *et al.*, *Mol. Cell. Endocrinol.*, 125:133-141 (1996); Walton, *et al.*, *J. Clin. Endocrinol. Metab.*, 86(8):3675-3685 (08/2001); Ulloa-Aguirre, *et al.*, *Endocrine*, 11(3):205-215 (12/1999); Castro-Fernández, *et al.*, *J. Clin. Endocrinol. Metab.*, 85(12):4603-4610 (2000); Prevost, Rebecca R., *Pharmacotherapy*, 18(5):1001-1010 (1998); Linskens, *et al.*, *The FASEB*

*Journal*, 13:639-645 (04/1999); Butnev, *et al.*, *Biol. Reprod.*, 58:458-469 (1998); Muyan, *et al.*, *Mol. Endo.*, 12(5):766-772 (1998); Min, *et al.*, *Endo. J.*, 43(5):585-593 (1996); Boime, *et al.*, *Recent Progress in Hormone Research*, 34:271-289 (1999); and Rafferty, *et al.*, *J. Endo.*, 145:527-533 (1995). The invention provides conjugates of glycosylated and unglycosylated peptides having follicle stimulating activity. The conjugates may be additionally modified by further conjugation with diverse species such as therapeutic moieties, diagnostic moieties, targeting moieties and the like.

The present invention further includes a method for remodeling and/or modifying FSH. Human reproductive function is controlled in part by a family of heterodimeric human glycoprotein hormones which have a common 92 amino acid glycoprotein alpha subunit, but differ in their hormone-specific beta subunits. The family includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin or thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). Human FSH and LH are used therapeutically to regulate various aspects of metabolism pertinent to reproduction in the human female. For example, FSH partially purified from urine is used clinically to stimulate follicular maturation in anovulatory women with anovulatory syndrome or luteal phase deficiency. Luteinizing hormone (LH) and FSH are used in combination to stimulate the development of ovarian follicles for *in vitro* fertilization. The role of FSH in the reproductive cycle is sufficiently well-known to permit therapeutic use, but difficulties have been encountered due, in part, to the heterogeneity and impurity of the preparation from native sources. This heterogeneity is due to variations in glycosylation pattern.

FSH is a valuable tool in both *in vitro* fertilization and stimulation of fertilization *in vivo*, but as stated above, its clinical efficacy has been hampered by inconsistency in glycosylation of the protein. It therefore seems apparent that a method for remodeling FSH will be of great benefit to the reproductive sciences.

A remodeled FSH peptide may be administered to a patient selected from the group consisting of a patient undergoing intrauterine insemination (IUI), a patient undergoing *in vitro* fertilization (IVF), and an infertile patient. A remodeled FSH peptide may also be administered to induce or increase ovulation in a patient, to stimulate development of an ovarian follicle in a patient, to induce gametogenic follicle growth in a patient, to stimulate, induce or increase follicle development and subsequent ovulation in a patient, or to treat infertility in a patient. Preferably, the patient is a human female patient. A remodeled FSH

peptide may also be administered to a patient having a pituitary deficiency or to a patient during puberty. Preferably this patient is a human male patient.

FSH has been cloned and sequenced, the nucleic and amino acid sequences of which are presented herein as SEQ ID NO:1. The skilled artisan will readily appreciate that 5 the present invention is not limited to the sequences depicted herein, as variants of FSH are well known in the art. As a non-limiting example, U.S. Patent No. 5,639,640 describes the beta subunit comprising two different amino acid sequences and U.S. Patent No. 5,338,835 describes a beta subunit comprising an additional amino acid sequence of approximately twenty-seven amino acids derived from the beta subunit of human chorionic gonadotropin. 10 Therefore, the present invention comprises FSH variants, both natural and engineered by the human hand, all well known in the art.

Methods to express FSH in cells, both prokaryotic and eukaryotic, are well known in the art and abundantly described in the literature (U.S. Patent Nos. 4,840,896, 4,923,805, 5,156,957). Further, methods for evaluating the biological activity of a remodeled 15 FSH molecule of the present invention are well known in the art, and are described in, for example, U.S. Patent No. 4,589,402, in which methods for determining the effect of FSH on fertility, egg production, and pregnancy rates is described in both non-human primates and human subjects.

The conjugates of the invention are formed by the enzymatic attachment of a 20 modified sugar to the glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide and the modifying group on the sugar becomes what is referred to herein as “an intact glycosyl linking group.” Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a 25 modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

In contrast to known chemical and enzymatic peptide elaboration strategies, 30 the methods of the invention, make it possible to assemble peptides and glycopeptides that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular amino acid residue or combination of amino acid residues of the peptide. The methods are also practical for large-scale production of modified peptides and glycopeptides. Thus, the methods of the invention provide a practical means for

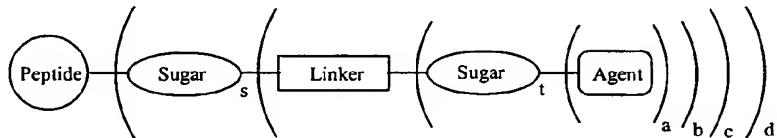
large-scale preparation of glycopeptides having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (e.g., mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or 5 prokaryotic cells) or transgenic plants or animals.

The present invention also provides conjugates of glycosylated and unglycosylated peptides with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants 10 on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent.

### The Conjugates

15 In a first aspect, the present invention provides a conjugate between a peptide and a selected moiety. The link between the peptide and the selected moiety includes an intact glycosyl linking group interposed between the peptide and the selected moiety. As discussed herein, the selected moiety is essentially any species that can be attached to a saccharide unit, resulting in a “modified sugar” that is recognized by an appropriate 20 transferase enzyme, which appends the modified sugar onto the peptide. The saccharide component of the modified sugar, when interposed between the peptide and a selected moiety, becomes an “intact glycosyl linking group.” The glycosyl linking group is formed from any mono- or oligo-saccharide that, after modification with a selected moiety, is a substrate for an appropriate transferase.

25 The conjugates of the invention will typically correspond to the general structure:



in which the symbols a, b, c, d and s represent a positive, non-zero integer; and t is either 0 or a positive integer. The “agent” is typically a PEG moiety. The linker can be any of a wide 30 array of linking groups, *infra*. Alternatively, the linker may be a single bond or a “zero order linker.”

Essentially any follicle stimulatory hormone or agent, having any sequence is of use as a component of the conjugates of the present invention. In an exemplary embodiment, the peptide has the sequence shown in **FIG. 6** (SEQ. ID NO.: 1). The three dimensional structure of the FSH peptide, with glycosylation sites identified is shown in  
5 **FIG.1.**

Preferably, neither terminus is derivatized.

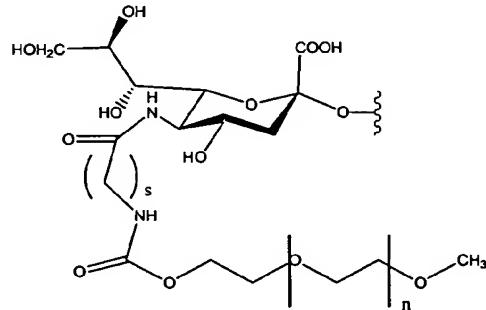
The peptides of the invention include at least one N-linked or O-linked glycosylation site, which is glycosylated with a glycosyl residue that includes a PEG moiety. The PEG is covalently attached to the peptide via an intact glycosyl linking group. The  
10 glycosyl linking group is covalently attached to either an amino acid residue or a glycosyl residue of the peptide. Alternatively, the glycosyl linking group is attached to one or more glycosyl units of a glycopeptide. The invention also provides conjugates in which the glycosyl linking group is attached to both an amino acid residue and a glycosyl residue.

The PEG moiety is attached to an intact glycosyl linker directly, or via a non-  
15 glycosyl linker, e.g., substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl.

In a preferred embodiment, at least one asparagine residue at N7, N24, N52, and N78 is derivatized, via an intact glycosyl linking group, with the sialic acid-PEG moiety shown in **FIG. 5**.

20 In the PEG moieties of use in the present invention, n is selected from the integers from 0 to about 500, more preferably from about 50 to about 300, and more preferably still from about 100 to about 300.

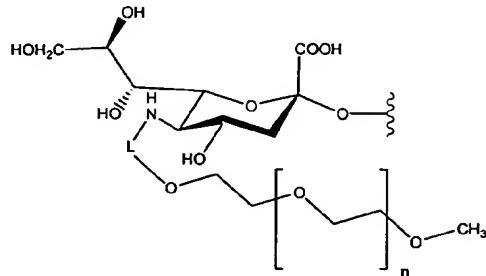
In another exemplary embodiment, the PEG-modified sialic acid moiety in the conjugate of the invention has the formula:



25

in which the index "s" represents an integer from 0 to 20.

In a still further exemplary embodiment, the PEG-modified sialic acid in has the formula:



in which L is a substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl

5 linker moiety joining the sialic acid moiety and the PEG moiety.

In a preferred embodiment, at least two, more preferably all three of the above-named asparagine residues is functionalized with the N-linked glycan chain shown above.

PEG moieties of any molecular weight, e.g., 5 Kd, 10 Kd, 20 Kd, are of use in  
10 the present invention. In a preferred embodiment, the PEG molecular weights are number-average molecular weights, which correlate closely to the peak-average molecular weight. Thus, glycans derivatized with 10 Kd PEG will have n ~ 230 (as shown in the structure above) at the center of its SEC peak, but there will be considerable variability in that chain length. In an exemplary embodiment, in the mid-to-high end of an exemplary PEG  
15 polydispersity profile, there will be measurable amounts of PEG with n~90 and n~360.

In addition to providing conjugates that are formed through an enzymatically added intact glycosyl linking group, the present invention provides conjugates that are highly homogenous in their substitution patterns. Using the methods of the invention, it is possible to form peptide conjugates in which essentially all of the modified sugar moieties across a  
20 population of conjugates of the invention are attached to multiple copies of a structurally identical amino acid or glycosyl residue. Thus, in a second aspect, the invention provides a peptide conjugate having a population of PEG moiety moieties, which are covalently bound to the peptide through an intact glycosyl linking group. In a preferred conjugate of the invention, essentially each member of the population is bound via the glycosyl linking group  
25 to a glycosyl residue of the peptide, and each glycosyl residue of the peptide to which the glycosyl linking group is attached has the same structure.

Also provided is a peptide conjugate having a population of PEG moieties covalently bound thereto through an intact glycosyl linking group. In a preferred

embodiment, essentially every member of the population of PEG moieties is bound to an amino acid residue of the peptide via an intact glycosyl linking group, and each amino acid residue having an intact glycosyl linking group attached thereto has the same structure.

The conjugates of the invention can include intact glycosyl linking groups that 5 are mono- or multi-valent (e.g., antennary structures). Thus, conjugates of the invention include both species in which a selected moiety is attached to a peptide via a monovalent glycosyl linking group. Also included within the invention are conjugates in which more than one selected moiety is attached to a peptide via a multivalent linking group.

## 10 The Methods

In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between a selected moiety and an FSH peptide. Additionally, the invention provides methods for targeting conjugates of the 15 invention to a particular tissue or region of the body.

In exemplary embodiments, the conjugate is formed between a PEG moiety (or an enzymatically transferable glycosyl moiety comprising the PEG moiety), and a glycosylated or non-glycosylated peptide. The PEG is conjugated to the peptide via an intact glycosyl linking group, which is interposed between, and covalently linked to both the 20 peptide and the PEG moiety, or to a PEG-non-glycosyl linker (e.g., substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl) construct. The method includes contacting the peptide with a mixture containing a modified sugar and a glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions sufficient to form a covalent bond between the modified sugar and the peptide. The sugar 25 moiety of the modified sugar is preferably selected from nucleotide sugars, activated sugars and sugars, which are neither nucleotides nor activated.

The acceptor peptide (glycosylated or non-glycosylated) is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (e.g., bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant 30 cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more N- or O-linked glycosylation sites to the peptide sequence.

The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced

glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, e.g., immunogenicity, recognition by the RES. Employing a modified sugar in a method of the invention, the peptide can be simultaneously further glycosylated and derivatized with, e.g., a PEG moiety, therapeutic agent, or the like. The sugar moiety of the modified sugar can be the residue that would properly be conjugated to the acceptor in a fully glycosylated peptide, or another sugar moiety with desirable properties.

FSH peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (e.g., N-acetylglactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to a the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

For example, in one embodiment, FSH is expressed in a mammalian system and modified by treatment of sialidase to trim back terminal sialic acid residues, followed by PEGylation using ST3Gal3 and a donor of PEG-sialic acid, such as that shown in **FIG. 5**. An exemplary scheme according to this description is shown in **FIG. 2A**. An SDS PAGE gel demonstrating the glycopeylation of the FSH peptide according to this method is shown in **FIG. 2B**.

In another exemplary embodiment, FSH expressed in mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor.

FSH expressed in a mammalian system can also be treated with sialidase and galactosidase to trim back its sialic acid and galactose residues, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid.

In a further exemplary embodiment, FSH is expressed in insect cells and modified in the following procedure: N-acetylglucosamine is first added to FSH using an

appropriate N-acetylglucosamine donor and one or more of GnT-I, II, IV, and V; FSH is then PEGylated using a donor of PEG-galactose and a galactosyltransferase.

FSH produced in yeast can also be glycopegylated. For example, FSH is first treated with endoglycanase to trim back the glycosyl groups, galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated with ST3Gal3 and a donor of PEG-sialic acid.

Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an -OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.*, Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751 (1994); Stemmer, *Nature* 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. *See, for example* WO 98/31826.

Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound

trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al.*, *Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al.*, *Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.* **138**: 350 (1987).

Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N- and O-glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulphydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

### Modified Sugars

Modified glycosyl donor species ("modified sugars") are preferably selected from modified sugar nucleotides, activated modified sugars and modified sugars that are simple saccharides that are neither nucleotides nor activated. Any desired carbohydrate structure can be added to a peptide using the methods of the invention. Typically, the structure will be a monosaccharide, but the present invention is not limited to the use of modified monosaccharide sugars; oligosaccharides and polysaccharides are useful as well.

The modifying group is attached to a sugar moiety by enzymatic means, chemical means or a combination thereof, thereby producing a modified sugar. The sugars are substituted at any position that allows for the attachment of the modifying moiety, yet which still allows the sugar to function as a substrate for the enzyme used to ligate the modified sugar to the peptide. In a preferred embodiment, when sialic acid is the sugar, the

sialic acid is substituted with the modifying group at either the 9-position on the pyruvyl side chain or at the 5-position on the amine moiety that is normally acetylated in sialic acid.

In certain embodiments of the present invention, a modified sugar nucleotide is utilized to add the modified sugar to the peptide. Exemplary sugar nucleotides that are used in the present invention in their modified form include nucleotide mono-, di- or tri-phosphates or analogs thereof. In a preferred embodiment, the modified sugar nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified sugar nucleotide is selected from an UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, or CMP-NeuAc.

10 N-acetylamine derivatives of the sugar nucleotides are also of use in the method of the invention.

The invention also provides methods for synthesizing a modified peptide using a modified sugar, *e.g.*, modified-galactose, -fucose, and -sialic acid. When a modified sialic acid is used, either a sialyltransferase or a trans-sialidase (for  $\alpha$ 2,3-linked sialic acid only) can be used in these methods.

In other embodiments, the modified sugar is an activated sugar. Activated modified sugars, which are useful in the present invention are typically glycosides which have been synthetically altered to include an activated leaving group. As used herein, the term "activated leaving group" refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions. Many activated sugars are known in the art. See, for example, Vocablo *et al.*, In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst *et al.* Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama *et al.*, *Tetrahedron Lett.* 34: 6419 (1993); Lougheed, *et al.*, *J. Biol. Chem.* 274: 37717 (1999)).

Examples of activating groups (leaving groups) include fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred activated leaving groups, for use in the present invention, are those that do not significantly sterically encumber the enzymatic transfer of the glycoside to the acceptor. Accordingly, preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides,  $\alpha$ -galactosyl fluoride,  $\alpha$ -mannosyl fluoride,  $\alpha$ -glucosyl fluoride,  $\alpha$ -fucosyl fluoride,  $\alpha$ -xylosyl fluoride,  $\alpha$ -sialyl fluoride,  $\alpha$ -N-acetylglucosaminyl fluoride,  $\alpha$ -N-acetylgalactosaminyl fluoride,  $\beta$ -galactosyl fluoride,  $\beta$ -mannosyl fluoride,  $\beta$ -glucosyl fluoride,  $\beta$ -fucosyl fluoride,  $\beta$ -xylosyl

fluoride,  $\beta$ -sialyl fluoride,  $\beta$ -N-acetylglucosaminyl fluoride and  $\beta$ -N-acetylgalactosaminyl fluoride are most preferred.

By way of illustration, glycosyl fluorides can be prepared from the free sugar by first acetylating the sugar and then treating it with HF/pyridine. This generates the 5 thermodynamically most stable anomer of the protected (acetylated) glycosyl fluoride (*i.e.*, the  $\alpha$ -glycosyl fluoride). If the less stable anomer (*i.e.*, the  $\beta$ -glycosyl fluoride) is desired, it can be prepared by converting the peracetylated sugar with HBr/HOAc or with HCl to generate the anomeric bromide or chloride. This intermediate is reacted with a fluoride salt such as silver fluoride to generate the glycosyl fluoride. Acetylated glycosyl fluorides may 10 be deprotected by reaction with mild (catalytic) base in methanol (*e.g.* NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available.

Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated hemiacetal form of the sugar with mesyl chloride, 15 followed by catalytic hydrogenation to remove the benzyl groups.

In a further exemplary embodiment, the modified sugar is an oligosaccharide having an antennary structure. In a preferred embodiment, one or more of the termini of the antennae bear the modifying moiety. When more than one modifying moiety is attached to an oligosaccharide having an antennary structure, the oligosaccharide is useful to “amplify” 20 the modifying moiety; each oligosaccharide unit conjugated to the peptide attaches multiple copies of the modifying group to the peptide. Many antennary saccharide structures are known in the art, and the present method can be practiced with them without limitation.

The poly(ethylene glycol) modifying group is discussed in greater detail below. The PEG moiety can be selected for one or more desirable property. Exemplary 25 properties include, but are not limited to, enhanced pharmacokinetics, enhanced pharmacodynamics, improved biodistribution, providing a polyvalent species, and improved water solubility.

### **Poly(ethylene glycol)**

The hydrodynamic radius and hydrophilicity of a selected peptide is enhanced 30 by conjugation with polar molecules such as poly(ethyleneglycol).

Methods and chemistry for activation of PEG moieties and saccharides as well as methods for conjugating saccharides and polymers to various species are described in the literature. Commonly used methods for activation of polymers include activation of

functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, etc. (see, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTALS AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND 5 CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

The use of reactive derivatives of PEG (or other linkers) to attach one or more 10 peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethyleneglycol) are available commercially and in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. *See*, Abuchowski 15 *et al.* *Cancer Biochem. Biophys.*, **7**: 175-186 (1984); Abuchowski *et al.*, *J. Biol. Chem.*, **252**: 3582-3586 (1977); Jackson *et al.*, *Anal. Biochem.*, **165**: 114-127 (1987); Koide *et al.*, *Biochem Biophys. Res. Commun.*, **111**: 659-667 (1983)), tresylate (Nilsson *et al.*, *Methods Enzymol.*, **104**: 56-69 (1984); Delgado *et al.*, *Biotechnol. Appl. Biochem.*, **12**: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann *et al.*, *Makromol. Chem.*, **182**: 1379-1384 (1981); Joppich *et al.*, *Makromol. Chem.*, **180**: 1381-1384 (1979); 20 Abuchowski *et al.*, *Cancer Biochem. Biophys.*, **7**: 175-186 (1984); Katreet *et al.* *Proc. Natl. Acad. Sci. U.S.A.*, **84**: 1487-1491 (1987); Kitamura *et al.*, *Cancer Res.*, **51**: 4310-4315 (1991); Bocci *et al.*, *Z. Naturforsch.*, **38C**: 94-99 (1983), carbonates (Zalipsky *et al.*, *POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS*, **25** Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky *et al.*, *Biotechnol. Appl. Biochem.*, **15**: 100-114 (1992); Veronese *et al.*, *Appl. Biochem. Biotech.*, **11**: 141-152 (1985)), imidazolyl formates (Beauchamp *et al.*, *Anal. Biochem.*, **131**: 25-33 (1983); Berger *et al.*, *Blood*, **71**: 1641-1647 (1988)), 4-dithiopyridines (Woghiren *et al.*, *Bioconjugate Chem.*, **4**: 314-318 (1993)), isocyanates (Byun *et al.*, *ASAIO Journal*, M649-M-653 (1992)) 30 and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki *et al.*, (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. *See*, Veronese, *et al.*, *Appl. Biochem. Biotechnol.*, **11**: 141-152 (1985).

Several reviews and monographs on the functionalization and conjugation of PEG are available. *See*, for example, Harris, *Macromol. Chem. Phys.* **C25**: 325-373 (1985);

Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002).

5 Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 10 4,412,989), ribonuclease and superoxide dismutase (Veronese *et al.*, *App. Biochem. Biotech.* **11**: 141-45 (1985)).

Preferred PEG moieties are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are “homodisperse.”

15 The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched.

The *in vivo* half-life of therapeutic glycopeptides can also be enhanced with PEG moieties such as polyethylene glycol (PEG). For example, chemical modification of proteins with PEG (PEGylation) increases their molecular size and decreases their surface-20 and functional group-accessibility, each of which are dependent on the size of the PEG attached to the protein. This results in an improvement of plasma half-lives and in proteolytic-stability, and a decrease in immunogenicity and hepatic uptake (Chaffee *et al.* *J. Clin. Invest.* **89**: 1643-1651 (1992); Pyatak *et al.* *Res. Commun. Chem. Pathol Pharmacol.* **29**: 113-127 (1980)). PEGylation of interleukin-2 has been reported to increase its antitumor 25 potency *in vivo* (Katre *et al.* *Proc. Natl. Acad. Sci. USA.* **84**: 1487-1491 (1987)) and PEGylation of a F(ab')2 derived from the monoclonal antibody A7 has improved its tumor localization (Kitamura *et al.* *Biochem. Biophys. Res. Commun.* **28**: 1387-1394 (1990)). Thus, in another preferred embodiment, the *in vivo* half-life of a peptide derivatized with a PEG 30 moiety by a method of the invention is increased relevant to the *in vivo* half-life of the non-derivatized peptide.

The increase in peptide *in vivo* half-life is best expressed as a range of percent increase in this quantity. The lower end of the range of percent increase is about 40%, about 60%, about 80%, about 100%, about 150% or about 200%. The upper end of the range is about 60%, about 80%, about 100%, about 150%, or more than about 250%.

In an exemplary embodiment, the present invention provides a PEGylated FSH (FIG. 1, FIG. 2 and FIG. 5).

### Preparation of Modified Sugars

5 In general, the sugar moiety or sugar moiety-linker cassette and the PEG or PEG-linker cassette groups are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or unreactive species. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present  
10 invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (e.g., reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (e.g., enamine reactions) and additions to carbon-carbon and  
15 carbon-heteroatom multiple bonds (e.g., Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society,  
20 Washington, D.C., 1982.

Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

- (a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;
- (b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*
- (c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;
- (d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkylolithium addition;

5 (f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

10 (h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see*, for 20 example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying 25 group is attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-30 acetylgalactosamine and fucose to name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* 6: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* 65: 24 (2000)).

In an exemplary embodiment, the peptide that is modified by a method of the invention is a glycopeptide that is produced in mammalian cells (e.g., CHO cells) or in a transgenic animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and containing a terminal galactose residue can be PEGylated, PPGylated or otherwise modified with a modified sialic acid.

In Scheme 1, the amino glycoside 1, is treated with the active ester of a protected amino acid (e.g., glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form  $\alpha$ -hydroxy carboxylate 2. Compound 2 is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound 3. The amine introduced via formation of the glycine adduct is utilized as a locus of PEG attachment by reacting compound 3 with an activated PEG or PPG derivative (e.g., PEG-C(O)NHS, PEG-OC(O)O-p-nitrophenyl), producing species such as 4 or 5, respectively.

**Scheme 1**

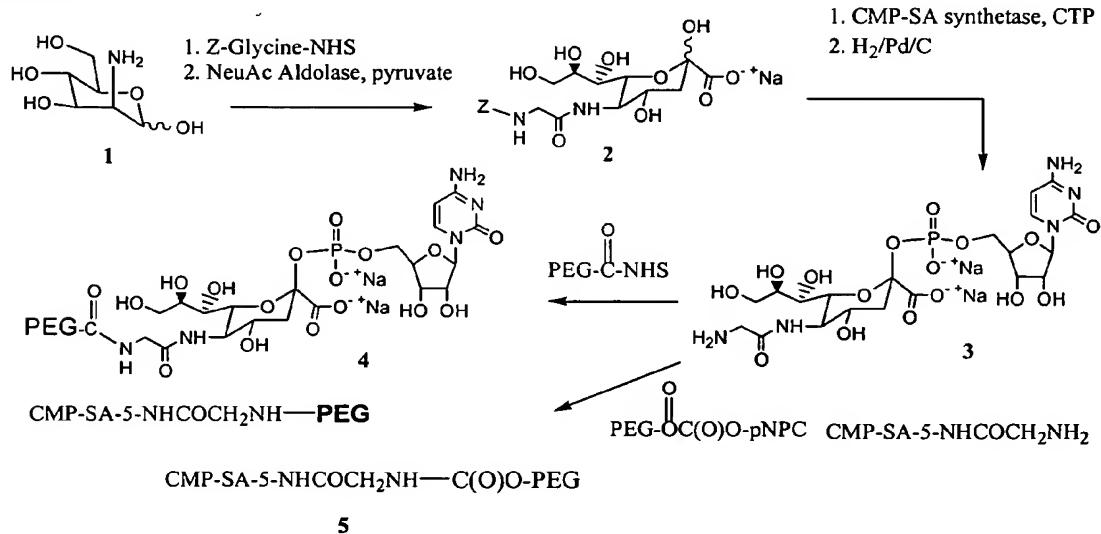
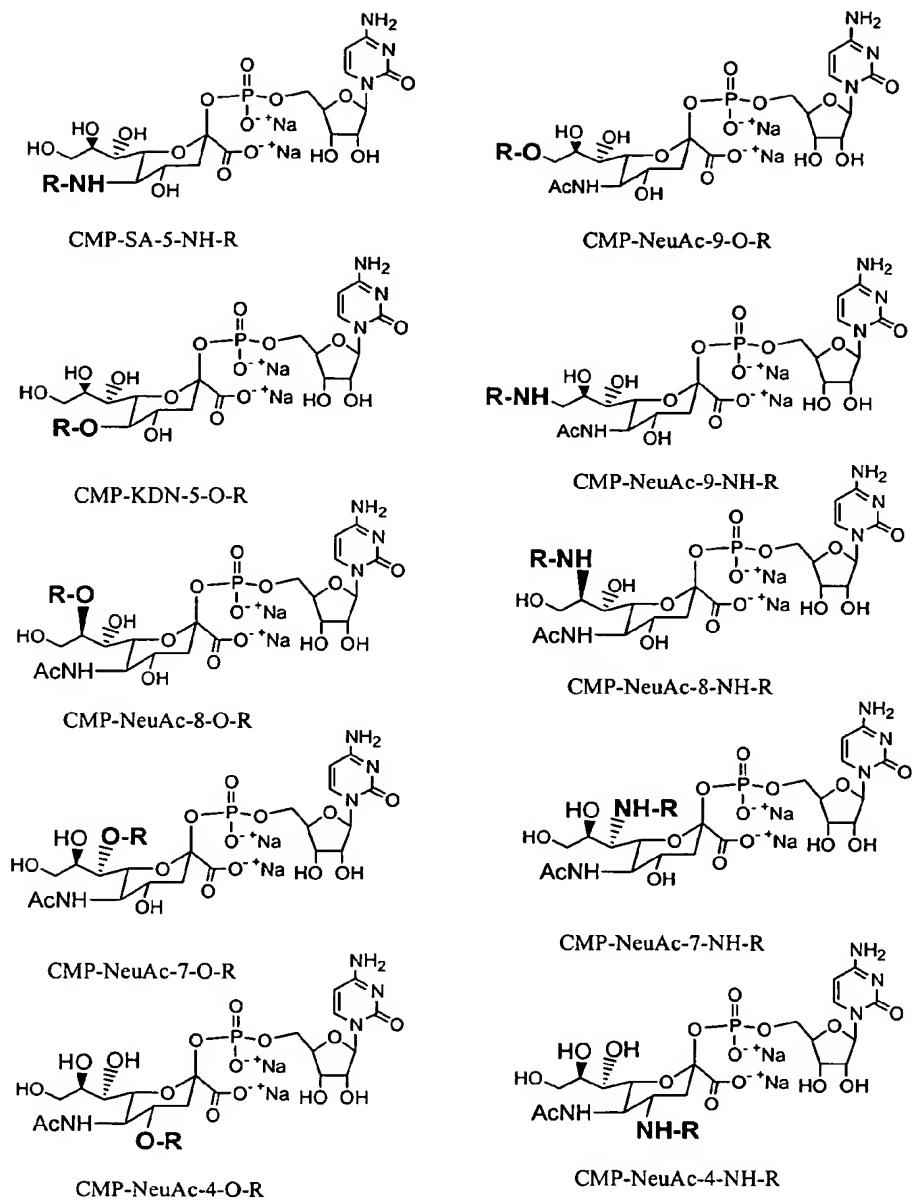
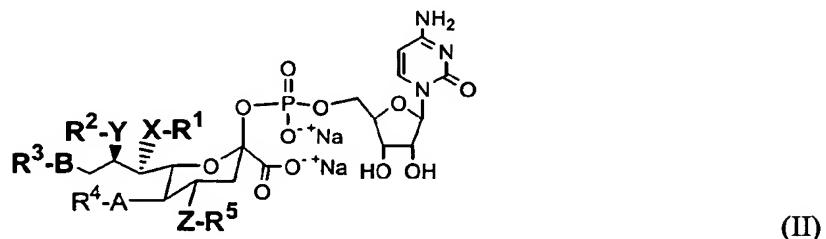


Table 2 sets forth representative examples of sugar monophosphates that are derivatized with a PEG moiety. Certain of the compounds of Table 1 are prepared by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. *See, for example, Keppler et al., Glycobiology 11: 11R (2001); and Charter et al., Glycobiology 10: 1049 (2000)).* Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of skill in the art.

**Table 1**



5 The modified sugar phosphates of use in practicing the present invention can be substituted in other positions as well as those set forth above. Presently preferred substitutions of sialic acid are set forth in Formula II:



in which X is a linking group, which is preferably selected from -O-, -N(H)-, -S, CH<sub>2</sub>-, and -N(R)<sub>2</sub>, in which each R is a member independently selected from R<sup>1</sup>-R<sup>5</sup>. The symbols Y, Z, A and B each represent a group that is selected from the group set forth above for the identity of X. X, Y, Z, A and B are each independently selected and, therefore, they can be the same or different. The symbols R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> represent H, a PEG moiety, therapeutic moiety, biomolecule or other moiety. Alternatively, these symbols represent a linker that is bound to a PEG moiety, therapeutic moiety, biomolecule or other moiety.

Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (e.g., acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (e.g., acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe<sub>x</sub>, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

### Linker Groups (Cross-linking Groups)

Preparation of the modified sugar for use in the methods of the present invention includes attachment of a PEG moiety to a sugar residue and preferably, forming a stable adduct, which is a substrate for a glycosyltransferase. Thus, it is often preferred to use a linker, e.g., one formed by reaction of the PEG and sugar moiety with a cross-linking agent to conjugate the PEG and the sugar. Exemplary bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethyleneglycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. See, for example, Lee *et al.*, *Biochemistry* **28**: 1856 (1989); Bhatia *et al.*, *Anal. Biochem.* **178**: 408 (1989); Janda *et al.*, *J. Am. Chem. Soc.* **112**: 8886 (1990) and Bednarski *et al.*, WO 92/18135.

In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

5 An exemplary strategy involves incorporation of a protected sulfhydryl onto the sugar using the heterobifunctional crosslinker SPDP (n-succinimidyl-3-(2-pyridyldithio)propionate and then deprotecting the sulfhydryl for formation of a disulfide bond with another sulfhydryl on the modifying group.

If SPDP detrimentally affects the ability of the modified sugar to act as a 10 glycosyltransferase substrate, one of an array of other crosslinkers such as 2-iminothiolane or N-succinimidyl S-acetylthioacetate (SATA) is used to form a disulfide bond. 2-iminothiolane reacts with primary amines, instantly incorporating an unprotected sulfhydryl onto the amine-containing molecule. SATA also reacts with primary amines, but incorporates a protected sulfhydryl, which is later deacetylated using hydroxylamine to 15 produce a free sulfhydryl. In each case, the incorporated sulfhydryl is free to react with other sulfhydryls or protected sulfhydryl, like SPDP, forming the required disulfide bond.

The above-described strategy is exemplary, and not limiting, of linkers of use 20 in the invention. Other crosslinkers are available that can be used in different strategies for crosslinking the modifying group to the peptide. For example, TPCH(S-(2-thiopyridyl)-L-cysteine hydrazide and TPMPH ((S-(2-thiopyridyl) mercapto-propionohydrazide) react with 25 carbohydrate moieties that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the crosslinker and the periodate generated aldehydes. TPCH and TPMPH introduce a 2-pyridylthione protected sulfhydryl group onto the sugar, which can be deprotected with DTT and then subsequently used for conjugation, such as forming disulfide bonds between components.

If disulfide bonding is found unsuitable for producing stable modified sugars, other crosslinkers may be used that incorporate more stable bonds between components. The heterobifunctional crosslinkers GMBS (N-gama-malimidobutyryloxy)succinimide) and SMCC (succinimidyl 4-(N-maleimido-methyl)cyclohexane) react with primary amines, thus 30 introducing a maleimide group onto the component. The maleimide group can subsequently react with sulfhydryls on the other component, which can be introduced by previously mentioned crosslinkers, thus forming a stable thioether bond between the components. If steric hindrance between components interferes with either component's activity or the ability of the modified sugar to act as a glycosyltransferase substrate, crosslinkers can be used which

introduce long spacer arms between components and include derivatives of some of the previously mentioned crosslinkers (*i.e.*, SPDP). Thus, there is an abundance of suitable crosslinkers, which are useful; each of which is selected depending on the effects it has on optimal peptide conjugate and modified sugar production.

5 A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* **25**: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcnenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* **91**: 580-609, 1983; Mattson *et al.*, *Mol. 10 Biol. Rep.* **17**: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example 15 is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide  $\gamma$ -glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer 20 reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulphydryl, guanidino, indole, or nonspecific groups.

25 *i. Preferred Specific Sites in Crosslinking Reagents*

*1. Amino-Reactive Groups*

In one preferred embodiment, the sites on the cross-linker are amino-reactive groups. Useful non-limiting examples of amino-reactive groups include N-hydroxysuccinimide (NHS) esters, imidoesters, isocyanates, acylhalides, arylazides, p-nitrophenyl esters, aldehydes, and sulfonyl chlorides.

NHS esters react preferentially with the primary (including aromatic) amino groups of a modified sugar component. The imidazole groups of histidines are known to compete with primary amines for reaction, but the reaction products are unstable and readily hydrolyzed. The reaction involves the nucleophilic attack of an amine on the acid carboxyl

of an NHS ester to form an amide, releasing the N-hydroxysuccinimide. Thus, the positive charge of the original amino group is lost.

5 Imidoesters are the most specific acylating reagents for reaction with the amine groups of the modified sugar components. At a pH between 7 and 10, imidoesters react only with primary amines. Primary amines attack imidates nucleophilically to produce an intermediate that breaks down to amidine at high pH or to a new imidate at low pH. The new imidate can react with another primary amine, thus crosslinking two amino groups, a case of a putatively monofunctional imidate reacting bifunctionally. The principal product of reaction with primary amines is an amidine that is a stronger base than the original amine.

10 The positive charge of the original amino group is therefore retained.

Isocyanates (and isothiocyanates) react with the primary amines of the modified sugar components to form stable bonds. Their reactions with sulphydryl, imidazole, and tyrosyl groups give relatively unstable products.

15 Acylazides are also used as amino-specific reagents in which nucleophilic amines of the affinity component attack acidic carboxyl groups under slightly alkaline conditions, *e.g.* pH 8.5.

Arylhalides such as 1,5-difluoro-2,4-dinitrobenzene react preferentially with the amino groups and tyrosine phenolic groups of modified sugar components, but also with sulphydryl and imidazole groups.

20 p-Nitrophenyl esters of mono- and dicarboxylic acids are also useful amino-reactive groups. Although the reagent specificity is not very high,  $\alpha$ - and  $\epsilon$ -amino groups appear to react most rapidly.

25 Aldehydes such as glutaraldehyde react with primary amines of modified sugar. Although unstable Schiff bases are formed upon reaction of the amino groups with the aldehydes of the aldehydes, glutaraldehyde is capable of modifying the modified sugar with stable crosslinks. At pH 6-8, the pH of typical crosslinking conditions, the cyclic polymers undergo a dehydration to form  $\alpha$ - $\beta$  unsaturated aldehyde polymers. Schiff bases, however, are stable, when conjugated to another double bond. The resonant interaction of both double bonds prevents hydrolysis of the Schiff linkage. Furthermore, amines at high local 30 concentrations can attack the ethylenic double bond to form a stable Michael addition product.

Aromatic sulfonyl chlorides react with a variety of sites of the modified sugar components, but reaction with the amino groups is the most important, resulting in a stable sulfonamide linkage.

5        **2. Sulphydryl-Reactive Groups**

In another preferred embodiment, the sites are sulphydryl-reactive groups. Useful, non-limiting examples of sulphydryl-reactive groups include maleimides, alkyl halides, pyridyl disulfides, and thiophthalimides.

10      Maleimides react preferentially with the sulphydryl group of the modified sugar components to form stable thioether bonds. They also react at a much slower rate with primary amino groups and the imidazole groups of histidines. However, at pH 7 the maleimide group can be considered a sulphydryl-specific group, since at this pH the reaction rate of simple thiols is 1000-fold greater than that of the corresponding amine.

15      Alkyl halides react with sulphydryl groups, sulfides, imidazoles, and amino groups. At neutral to slightly alkaline pH, however, alkyl halides react primarily with sulphydryl groups to form stable thioether bonds. At higher pH, reaction with amino groups is favored.

20      Pyridyl disulfides react with free sulphydryls via disulfide exchange to give mixed disulfides. As a result, pyridyl disulfides are the most specific sulphydryl-reactive groups.

Thiophthalimides react with free sulphydryl groups to form disulfides.

3. **Carboxyl-Reactive Residue**

25      In another embodiment, carbodiimides soluble in both water and organic solvent, are used as carboxyl-reactive reagents. These compounds react with free carboxyl groups forming a pseudourea that can then couple to available amines yielding an amide linkage (teach how to modify a carboxyl group with carbodiimde (Yamada *et al.*, *Biochemistry* **20**: 4836-4842, 1981).

30        ***ii. Preferred Nonspecific Sites in Crosslinking Reagents***

In addition to the use of site-specific reactive moieties, the present invention contemplates the use of non-specific reactive groups to link the sugar to the modifying group.

Exemplary non-specific cross-linkers include photoactivatable groups, completely inert in the dark, which are converted to reactive species upon absorption of a

photon of appropriate energy. In one preferred embodiment, photoactivatable groups are selected from precursors of nitrenes generated upon heating or photolysis of azides. Electron-deficient nitrenes are extremely reactive and can react with a variety of chemical bonds including N-H, O-H, C-H, and C=C. Although three types of azides (aryl, alkyl, and acyl derivatives) may be employed, arylazides are presently preferred. The reactivity of arylazides upon photolysis is better with N-H and O-H than C-H bonds. Electron-deficient arylnitrenes rapidly ring-expand to form dehydroazepines, which tend to react with nucleophiles, rather than form C-H insertion products. The reactivity of arylazides can be increased by the presence of electron-withdrawing substituents such as nitro or hydroxyl groups in the ring. Such substituents push the absorption maximum of arylazides to longer wavelength. Unsubstituted arylazides have an absorption maximum in the range of 260-280 nm, while hydroxy and nitroarylazides absorb significant light beyond 305 nm. Therefore, hydroxy and nitroarylazides are most preferable since they allow to employ less harmful photolysis conditions for the affinity component than unsubstituted arylazides.

15 In another preferred embodiment, photoactivatable groups are selected from fluorinated arylazides. The photolysis products of fluorinated arylazides are arylnitrenes, all of which undergo the characteristic reactions of this group, including C-H bond insertion, with high efficiency (Keana *et al.*, *J. Org. Chem.* **55**: 3640-3647, 1990).

20 In another embodiment, photoactivatable groups are selected from benzophenone residues. Benzophenone reagents generally give higher crosslinking yields than arylazide reagents.

25 In another embodiment, photoactivatable groups are selected from diazo compounds, which form an electron-deficient carbene upon photolysis. These carbenes undergo a variety of reactions including insertion into C-H bonds, addition to double bonds (including aromatic systems), hydrogen attraction and coordination to nucleophilic centers to give carbon ions.

30 In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like formaldehyde or glutaraldehyde forming crosslinks.

*iii. Homobifunctional Reagents*

**1. Homobifunctional crosslinkers reactive with primary amines**

Synthesis, properties, and applications of amine-reactive cross-linkers are commercially described in the literature (for reviews of crosslinking procedures and reagents, 5 *see above*). Many reagents are available (e.g., Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR.).

Preferred, non-limiting examples of homobifunctional NHS esters include disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS), disuccinimidyl tartarate (DST), disulfosuccinimidyl tartarate (sulfo-DST), bis-10 2-(succinimidooxycarbonyloxy)ethylsulfone (BSOCOES), bis-2-(sulfosuccinimidooxy- carbonyloxy)ethylsulfone (sulfo-BSOCOES), ethylene glycolbis(succinimidylsuccinate) (EGS), ethylene glycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS), dithiobis(succinimidylpropionate) (DSP), and dithiobis(sulfosuccinimidylpropionate) (sulfo-DSP). Preferred, non-limiting examples of homobifunctional imidoesters include dimethyl malonimidate (DMM), 15 dimethyl succinimidate (DMSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3'-oxydipropionimidate (DODP), dimethyl-3,3'-(methylenedioxy)dipropionimidate (DMDP), dimethyl-3,3'-(dimethylenedioxy)dipropionimidate (DDDP), dimethyl-3,3'-(tetramethylenedioxy)- dipropionimidate (DTDP), and dimethyl-3,3'-dithiobispropionimidate (DTBP).

20 Preferred, non-limiting examples of homobifunctional isothiocyanates include: p-phenylenediisothiocyanate (DITC), and 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS).

Preferred, non-limiting examples of homobifunctional isocyanates include xylene-diisocyanate, toluene-2,4-diisocyanate, toluene-2-isocyanate-4-isothiocyanate, 3-25 methoxydiphenylmethane-4,4'-diisocyanate, 2,2'-dicarboxy-4,4'-azophenyldiisocyanate, and hexamethylenediisocyanate.

Preferred, non-limiting examples of homobifunctional arylhalides include 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and 4,4'-difluoro-3,3'-dinitrophenyl-sulfone.

30 Preferred, non-limiting examples of homobifunctional aliphatic aldehyde reagents include glyoxal, malondialdehyde, and glutaraldehyde.

Preferred, non-limiting examples of homobifunctional acylating reagents include nitrophenyl esters of dicarboxylic acids.

Preferred, non-limiting examples of homobifunctional aromatic sulfonyl chlorides include phenol-2,4-disulfonyl chloride, and  $\alpha$ -naphthol-2,4-disulfonyl chloride.

Preferred, non-limiting examples of additional amino-reactive homobifunctional reagents include erythritolbiscarbonate which reacts with amines to give 5 biscarbamates.

## **2. Homobifunctional Crosslinkers Reactive with Free Sulfhydryl Groups**

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the 10 reagents are commercially available (e.g., Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional maleimides include bismaleimidohexane (BMH), N,N'-(1,3-phenylene) bismaleimide, N,N'-(1,2-phenylene)bismaleimide, azophenyldimaleimide, and bis(N-maleimidomethyl)ether.

15 Preferred, non-limiting examples of homobifunctional pyridyl disulfides include 1,4-di-3'-(2'-pyridylidithio)propionamidobutane (DPDPB).

Preferred, non-limiting examples of homobifunctional alkyl halides include 2,2'-dicarboxy-4,4'-diiodoacetamidoazobenzene,  $\alpha$ , $\alpha'$ -diiodo-p-xylenesulfonic acid,  $\alpha$ ,  $\alpha'$ -dibromo-p-xylenesulfonic acid, N,N'-bis(b-bromoethyl)benzylamine, N,N'-di(bromoacetyl)phenylhydrazine, and 1,2-di(bromoacetyl)amino-3-phenylpropane.

## **3. Homobifunctional Photoactivatable Crosslinkers**

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Some of the 25 reagents are commercially available (e.g., Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional photoactivatable crosslinker include bis- $\beta$ -(4-azidosalicylamido)ethyldisulfide (BASED), di-N-(2-nitro-4-azidophenyl)-cystamine-S,S-dioxide (DNCO), and 4,4'-dithiobisphenylazide.

iv. *HeteroBifunctional Reagents*

1. *Amino-Reactive HeteroBifunctional Reagents with a Pyridyl Disulfide Moiety*

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (e.g., Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of hetero-bifunctional reagents with a pyridyl disulfide moiety and an amino-reactive NHS ester include N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl 6-3-(2-pyridyldithio)propionamido hexanoate (LC-SPDP), sulfosuccinimidyl 6-3-(2-pyridyldithio)propionamido hexanoate (sulfo-LCSPDP), 4-succinimidyl oxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluene (SMPT), and sulfosuccinimidyl 6- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamido hexanoate (sulfo-LC-SMPT).

15 2. *Amino-Reactive HeteroBifunctional Reagents with a Maleimide Moiety*

Synthesis, properties, and applications of such reagents are described in the literature. Preferred, non-limiting examples of hetero-bifunctional reagents with a maleimide moiety and an amino-reactive NHS ester include succinimidyl maleimidyl acetate (AMAS), succinimidyl 3-maleimidyl propionate (BMPS), N- $\gamma$ -maleimidobutyryloxy succinimide ester (GMBS)N- $\gamma$ -maleimidobutyryloxy sulfo succinimide ester (sulfo-GMBS) succinimidyl 6-maleimidyl hexanoate (EMCS), succinimidyl 3-maleimidyl benzoate (SMB), m-maleimidobenzoyl-N-hydroxy succinimide ester (MBS), m-maleimidobenzoyl-N-hydroxy sulfo succinimide ester (sulfo-MBS), succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), and sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB).

30 3. *Amino-Reactive HeteroBifunctional Reagents with an Alkyl Halide Moiety*

Synthesis, properties, and applications of such reagents are described in the literature. Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive NHS ester include N-succinimidyl-(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl-(4-iodoacetyl)aminobenzoate (sulfo-

SIAB), succinimidyl-6-(iodoacetyl)aminohexanoate (SIAX), succinimidyl-6-(6-((iodoacetyl)-amino)hexanoylaminohexanoate (SIAXX), succinimidyl-6-(((4-(iodoacetyl)-amino)-methyl)-cyclohexane-1-carbonyl)aminohexanoate (SIACX), and succinimidyl-4((iodoacetyl)-amino)methylcyclohexane-1-carboxylate (SIAC).

5 A preferred example of a hetero-bifunctional reagent with an amino-reactive NHS ester and an alkyl dihalide moiety is N-hydroxysuccinimidyl 2,3-dibromopropionate (SDBP). SDBP introduces intramolecular crosslinks to the affinity component by conjugating its amino groups. The reactivity of the dibromopropionyl moiety towards primary amine groups is controlled by the reaction temperature (McKenzie *et al.*, *Protein Chem.* 7: 581-592 (1988)).

10 Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive p-nitrophenyl ester moiety include p-nitrophenyl iodoacetate (NPIA).

15 Other cross-linking agents are known to those of skill in the art. *See, for example*, Pomato *et al.*, U.S. Patent No. 5,965,106. It is within the abilities of one of skill in the art to choose an appropriate cross-linking agent for a particular application.

#### *v. Cleavable Linker Groups*

20 In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleavable groups are known in the art. *See, for example*, Jung *et al.*, *Biochem. Biophys. Acta* 761: 152-162 (1983); Joshi *et al.*, *J. Biol. Chem.* 265: 14518-14525 (1990); Zarling *et al.*, *J. Immunol.* 124: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.* 155: 141-147 (1986); Park *et al.*, *J. Biol. Chem.* 261: 205-210 (1986); Browning *et al.*, *J. Immunol.* 143: 1859-1867 (1989). Moreover 25 a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

Exemplary cleavable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytized (e.g., cis-aconityl; *see*, Shen *et al.*, *Biochem. Biophys. Res. Commun.* 102: 1048 (1991)). Preferred cleavable groups comprise a cleavable moiety which is a member selected from the group consisting of disulfide, ester, imide, carbonate, nitrobenzyl, phenacyl and benzoin groups.

## Conjugation of Modified Sugars to Peptides

The PEG modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected 5 such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

A number of methods of using glycosyltransferases to synthesize desired oligosaccharide structures are known and are generally applicable to the instant invention. 10 Exemplary methods are described, for instance, WO 96/32491, Ito *et al.*, *Pure Appl. Chem.* 65: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a galactosyltransferase. In those embodiments using more than one 15 enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over 20 procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure 25 on the peptide at any point either before or after the addition of the modified sugar to the peptide.

In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than rupture them. The mutant glycanase typically includes a substitution of an 30 amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine.

The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these embodiments, the glycosyl donor molecule (*e.g.*, a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

10 In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction 15 conditions are well known to those of skill in the art.

20 The temperature at which an above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 °C to about 30 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

25 The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (*e.g.*, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected 30 system.

The present invention also provides for the industrial-scale production of modified peptides. As used herein, an industrial scale generally produces at least one gram of finished, purified conjugate.

In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with PEG. The focus of the following discussion on the use of PEG-modified sialic acid and glycosylated peptides is for clarity of illustration and is not intended to imply

that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than PEG including other PEG moieties, therapeutic moieties, and biomolecules.

An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Gal $\beta$ 1,4GlcNAc, Gal $\beta$ 1,4GalNAc, Gal $\beta$ 1,3GalNAc, lacto-N-tetraose, Gal $\beta$ 1,3GlcNAc, Gal $\beta$ 1,3Ara, Gal $\beta$ 1,6GlcNAc, Gal $\beta$ 1,4Glc (lactose), and other acceptors known to those of skill in the art (see, e.g., Paulson *et al.*, *J. Biol. Chem.* **253**: 5617-5624 (1978)).

In one embodiment, an acceptor for the sialyltransferase is present on the glycopeptide to be modified upon *in vivo* synthesis of the glycopeptide. Such glycopeptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the glycopeptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, e.g., a GlcNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (e.g., gal $\beta$ 1,3 or gal $\beta$ 1,4), and a suitable galactosyl donor (e.g., UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the

galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

In yet another embodiment, glycopeptide-linked oligosaccharides are first “trimmed,” either in whole or in part, to expose either an acceptor for the sialyltransferase or 5 a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (see, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions.

In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a PEG moiety attached thereto. The focus of the 10 discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic moiety, biomolecule or the like.

In an exemplary embodiment of the invention in which a carbohydrate residue is “trimmed” prior to the addition of the modified sugar high mannose is trimmed back to the 15 first generation biantennary structure. A modified sugar bearing a PEG moiety is conjugated to one or more of the sugar residues exposed by the “trimming back.” In one example, a PEG moiety is added via a GlcNAc moiety conjugated to the PEG moiety. The modified GlcNAc is attached to one or both of the terminal mannose residues of the biantennary structure. Alternatively, an unmodified GlcNAc can be added to one or both of the termini of the 20 branched species.

In another exemplary embodiment, a PEG moiety is added to one or both of the terminal mannose residues of the biantennary structure via a modified sugar having a galactose residue, which is conjugated to a GlcNAc residue added onto the terminal mannose residues. Alternatively, an unmodified Gal can be added to one or both terminal GlcNAc 25 residues.

In yet a further example, a PEG moiety is added onto a Gal residue using a modified sialic acid.

In another exemplary, a high mannose structure is “trimmed back” to the mannose from which the biantennary structure branches. In one example, a PEG moiety is 30 added via a GlcNAc modified with the polymer. Alternatively, an unmodified GlcNAc is added to the mannose, followed by a Gal with an attached PEG moiety. In yet another embodiment, unmodified GlcNAc and Gal residues are sequentially added to the mannose, followed by a sialic acid moiety modified with a PEG moiety.

In a further exemplary embodiment, high mannose is “trimmed back” to the GlcNAc to which the first mannose is attached. The GlcNAc is conjugated to a Gal residue bearing a PEG moiety. Alternatively, an unmodified Gal is added to the GlcNAc, followed by the addition of a sialic acid modified with a water-soluble sugar. In yet a further example, 5 the terminal GlcNAc is conjugated with Gal and the GlcNAc is subsequently fucosylated with a modified fucose bearing a PEG moiety.

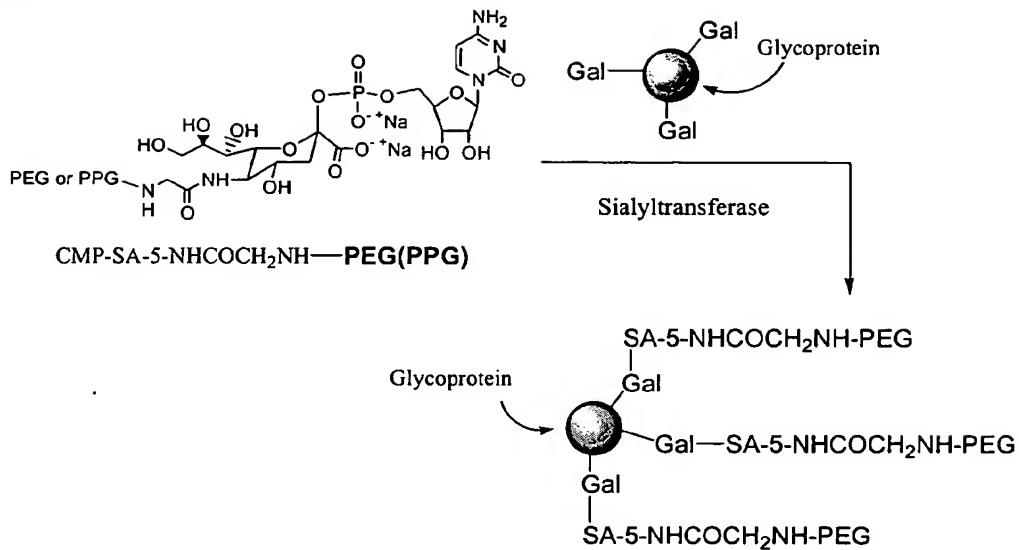
High mannose may also be trimmed back to the first GlcNAc attached to the Asn of the peptide. In one example, the GlcNAc of the GlcNAc-(Fuc)<sub>a</sub> residue is conjugated with a GlcNAc bearing a water soluble polymer. In another example, the GlcNAc of the 10 GlcNAc-(Fuc)<sub>a</sub> residue is modified with Gal, which bears a water soluble polymer. In a still further embodiment, the GlcNAc is modified with Gal, followed by conjugation to the Gal of a sialic acid modified with a PEG moiety.

Other exemplary embodiments are set forth in commonly owned patent applications 10/369,979; 10/410,913; 10/411,026; 10/287,994; 10/360,770; 10/410,945 and 15 PCT/US02/32263.

The Examples set forth above provide an illustration of the power of the methods set forth herein. Using the methods described herein, it is possible to “trim back” and build up a carbohydrate residue of substantially any desired structure. The modified sugar can be added to the termini of the carbohydrate moiety as set forth above, or it can be 20 intermediate between the peptide core and the terminus of the carbohydrate.

In an exemplary embodiment, an existing sialic acid is removed from a glycopeptide using a sialidase, thereby unmasking all or most of the underlying galactosyl residues. Alternatively, a peptide or glycopeptide is labeled with galactose residues, or an oligosaccharide residue that terminates in a galactose unit. Following the exposure of or 25 addition of the galactose residues, an appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 2.

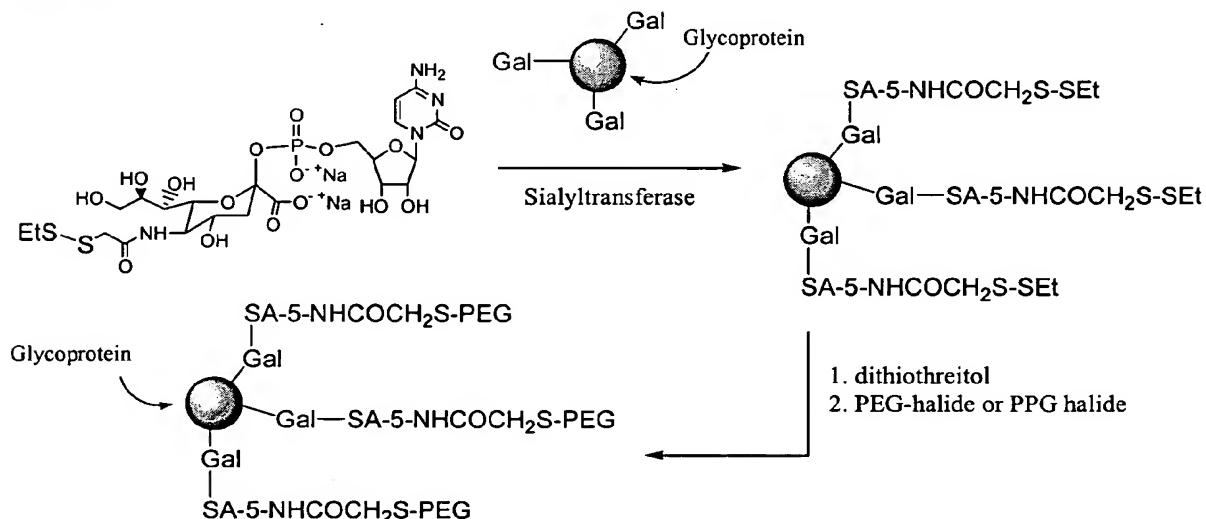
Scheme 2



In yet a further approach, summarized in Scheme 3, a masked reactive

5 functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the FSH. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

10 Scheme 3



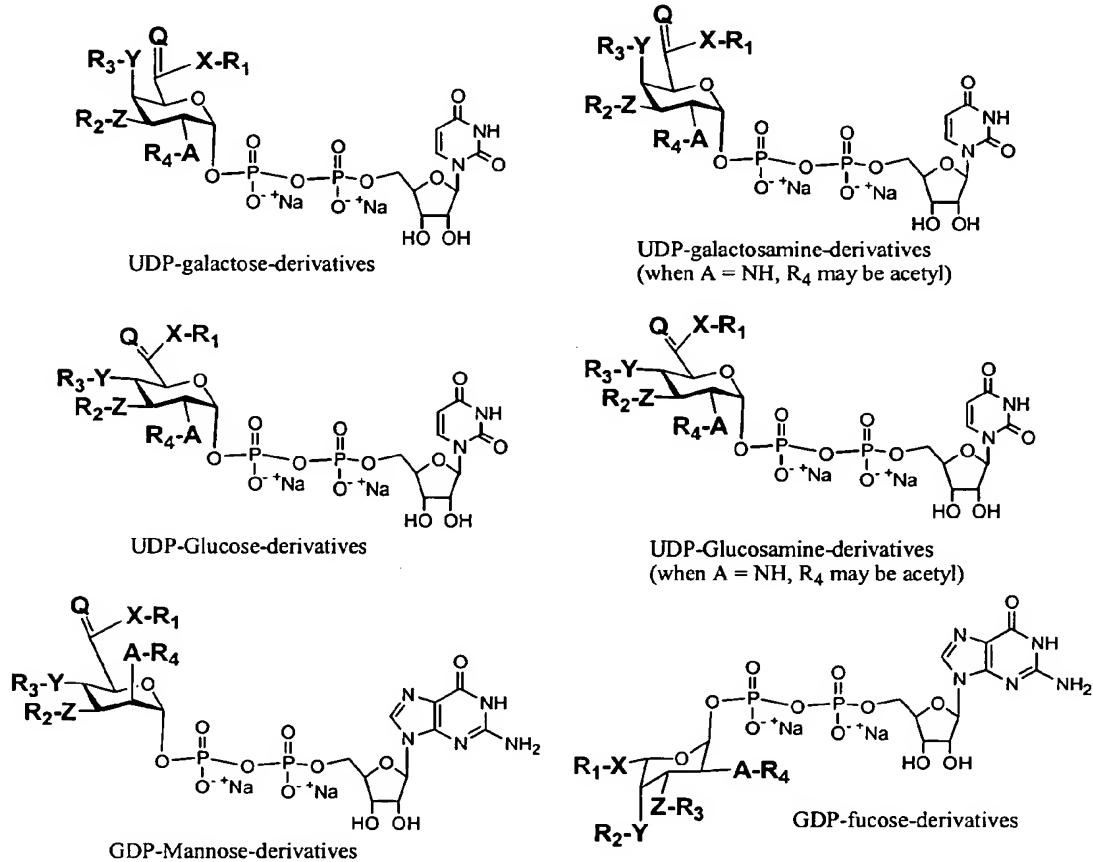
Any modified sugar can be used with its appropriate glycosyltransferase,

depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide

15 (Table 2). As discussed above, the terminal sugar of the glycopeptide required for

introduction of the PEGylated structure can be introduced naturally during expression or it can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix of glycosidase(s) and glycosyltransferase(s).

5 **Table 2**



$X = O, NH, S, CH_2, N-(R_{1-5})_2$ .  
 $Y = X; Z = X; A = X; B = X$ .  
 $Q = H_2, O, S, NH, N-R$ .  
 $R, R_{1-4} = H, \text{Linker-}M, M$ .  
 $M = \text{PEG, e.g., m-PEG}$

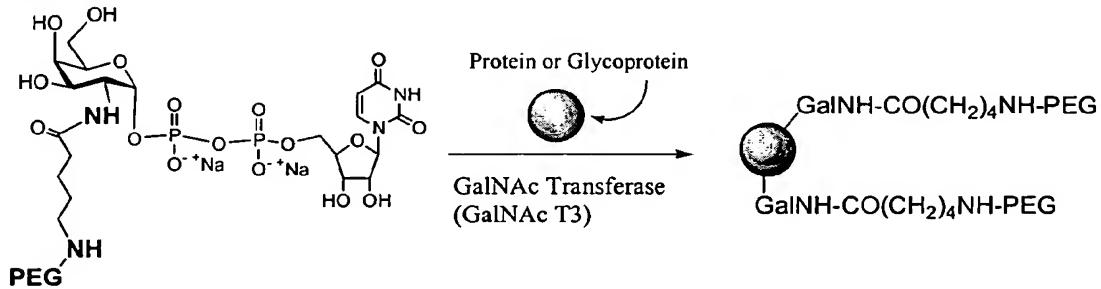
In a further exemplary embodiment, UDP-galactose-PEG is reacted with 10 bovine milk  $\beta$ 1,4-galactosyltransferase, thereby transferring the modified galactose to the appropriate terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide may be produced during expression, as may occur in such expression systems as

mammalian, insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase and/or glycosidase and/or glycosyltransferase, as required.

In another exemplary embodiment, a GlcNAc transferase, such as GNT1-5, is utilized to transfer PEGylated-GlcN to a terminal mannose residue on a glycopeptide. In a 5 still further exemplary embodiment, an the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the 10 appropriate galactosyltransferase is used to introduce the PEG-galactose functionality onto the exposed GlcNAc.

In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 4. Exemplary 15 glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-14), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the 20 modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the polypeptide 25 chain.

**Scheme 4**



In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the modified sugar to the peptide. In an exemplary embodiment, an enzyme (e.g., fucosyltransferase) is used to append a glycosyl unit (e.g., fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

15

*i. Enzymes*

*1. Glycosyltransferases*

Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-NAG<sub>2</sub>Glc<sub>3</sub>Man<sub>9</sub> in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

25 The glycosyltransferase to be used in the present invention may be any as long as it can utilize the modified sugar as a sugar donor. Examples of such enzymes include Leloir pathway glycosyltransferase, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g., "The WWW Guide To Cloned Glycosyltransferases,"* ([http://www.vei.co.uk/TGN/gt\\_guide.htm](http://www.vei.co.uk/TGN/gt_guide.htm)).

Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

Glycosyltransferases that can be employed in the methods of the invention

5 include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

10 DNA encoding the enzyme glycosyltransferases may be obtained by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the glycosyltransferases gene sequence. Probes may be labeled with a  
15 detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays. In the alternative, glycosyltransferases gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the glycosyltransferases gene sequence. *See*, U.S. Pat. No. 4,683,195 to Mullis *et al.* and U.S.  
20 Pat. No. 4,683,202 to Mullis.

The glycosyltransferases enzyme may be synthesized in host cells transformed with vectors containing DNA encoding the glycosyltransferases enzyme. A vector is a replicable DNA construct. Vectors are used either to amplify DNA encoding the glycosyltransferases enzyme and/or to express DNA which encodes the glycosyltransferases  
25 enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the glycosyltransferases enzyme is operably linked to suitable control sequences capable of effecting the expression of the glycosyltransferases enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional  
30 promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

25           a)       *Fucosyltransferases*

In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art.

5     Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ - group in an oligosaccharide glycoside. Suitable fucosyltransferases 10 for this reaction include the Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ 1- $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (see, Palcic, *et al.*, *Carbohydrate Res.* **190**: 1-11 (1989); Prieels, *et al.*, *J. Biol. Chem.* **256**: 10456-10463 (1981); and Nunez, *et al.*, *Can. J. Chem.* **59**: 2086-2095 (1981)) and the Gal $\beta$ (1 $\rightarrow$ 4)GlcNAc $\beta$ - $\alpha$ fucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 15 2.4.1.65), a sialyl  $\alpha$ (2 $\rightarrow$ 3)Gal $\beta$ ((1 $\rightarrow$ 3)GlcNAc $\beta$  fucosyltransferase, has also been characterized. A recombinant form of the Gal $\beta$ (1 $\rightarrow$ 3,4) GlcNAc $\beta$ - $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase has also been characterized (see, Dumas, *et al.*, *Bioorg. Med. Letters* **1**: 425-428 (1991) and Kukowska-Latallo, *et al.*, *Genes and Development* **4**: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example,  $\alpha$ 1,2 20 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al.*, *Eur. J. Biochem.* **191**: 169-176 (1990) or U.S. Patent No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

25           b)       *Galactosyltransferases*

In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include  $\alpha$ (1,3) galactosyltransferases (E.C. No. 2.4.1.151, see, e.g., Dabkowski *et al.*, *Transplant Proc.* **25**:2921 (1993) and Yamamoto *et al.* *Nature* **345**: 229-233 (1990), bovine (GenBank j04989, Joziasse *et al.*, *J. Biol. Chem.* **264**: 14290-14297 (1989)), murine (GenBank m26925; Larsen *et al.*, *Proc. Nat'l. Acad. Sci. USA* **86**: 8227-8231 (1989)), porcine (GenBank L36152; Strahan *et al.*, *Immunogenetics* **41**: 101-105 (1995)). Another suitable  $\alpha$ 1,3 galactosyltransferase is that

which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al.*, *J. Biol. Chem.* **265**: 1146-1151 (1990) (human)).

Also suitable for use in the methods of the invention are  $\beta(1,4)$  galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 5 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al.*, *Eur. J. Biochem.* **183**: 211-217 (1989)), human (Masri *et al.*, *Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.*, *J. Biochem.* **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al.*, *J. Neurosci. Res.* **38**: 234-242 (1994)). Other suitable galactosyltransferases include, for example,  $\alpha 1,2$  10 galactosyltransferases (from *e.g.*, *Schizosaccharomyces pombe*, Chapell *et al.*, *Mol. Biol. Cell* **5**: 519-528 (1994)).

The production of proteins such as the enzyme GalNAc T<sub>I-XIV</sub> from cloned genes by genetic engineering is well known. See, e.g., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is 15 determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies 20 of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (e.g., a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al.*, *Glycobiology* 6: v-xiv (1996)). An exemplary  $\alpha$ (2,3)sialyltransferase referred to as  $\alpha$ (2,3)sialyltransferase (EC

2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal $\beta$ 1 $\rightarrow$ 3Glc disaccharide or glycoside. *See*, Van den Eijnden *et al.*, *J. Biol. Chem.* **256**: 3159 (1981), Weinstein *et al.*, *J. Biol. Chem.* **257**: 13845 (1982) and Wen *et al.*, *J. Biol. Chem.* **267**: 21011 (1992). Another exemplary  $\alpha$ 2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see*, Rearick *et al.*, *J. Biol. Chem.* **254**: 4444 (1979) and Gillespie *et al.*, *J. Biol. Chem.* **267**: 21004 (1992). Further exemplary enzymes include Gal- $\beta$ -1,4-GlcNAc  $\alpha$ -2,6 sialyltransferase (*See*, Kurosawa *et al.* *Eur. J. Biochem.* **219**: 375-381 (1994)).

5 Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Gal $\beta$ 1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see*, Table 3).

10 **Table 3: Sialyltransferases which use the Gal $\beta$ 1,4GlcNAc sequence as an acceptor substrate**

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAcI2,6Gal $\beta$ 1,4GlcNAc-	1
ST3Gal III	Mammalian	NeuAcI2,3Gal $\beta$ 1,4GlcNAc- NeuAcI2,3Gal $\beta$ 1,3GlcNAc-	1
ST3Gal IV	Mammalian	NeuAcI2,3Gal $\beta$ 1,4GlcNAc- NeuAcI2,3Gal $\beta$ 1,3GlcNAc-	1
ST6Gal II	Mammalian	NeuAcI2,6Gal $\beta$ 1,4GlcNA	
ST6Gal II	photobacterium	NeuAcI2,6Gal $\beta$ 1,4GlcNAc-	2
ST3Gal V	<i>N. meningitidis</i> <i>N. gonorrhoeae</i>	NeuAcI2,3Gal $\beta$ 1,4GlcNAc-	3

15 1) Goochee *et al.*, *Bio/Technology* **9**: 1347-1355 (1991)

2) Yamamoto *et al.*, *J. Biochem.* **120**: 104-110 (1996)

3) Gilbert *et al.*, *J. Biol. Chem.* **271**: 28271-28276 (1996)

20 An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as  $\alpha$ (2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal $\beta$ 1,3GlcNAc or Gal $\beta$ 1,4GlcNAc glycoside (*see*, e.g., Wen *et al.*, *J. Biol. Chem.* **267**: 21011 (1992); Van den Eijnden *et al.*, *J. Biol. Chem.* **256**: 3159 (1991)) and is responsible for sialylation of asparagine-linked

oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an  $\alpha$ -linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein *et al.*, *J. Biol. Chem.* **257**: 13845 (1982)); the human cDNA (Sasaki *et al.* (1993) *J. Biol. Chem.* **268**: 22782-22787; Kitagawa & Paulson (1994) *J. Biol. Chem.* **269**: 1394-1401) and genomic (Kitagawa *et al.* (1996) *J. Biol. Chem.* **271**: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

Other exemplary sialyltransferases of use in the present invention include 10 those isolated from *Campylobacter jejuni*, including the  $\alpha(2,3)$ . *See, e.g.*, WO99/49051.

Other sialyltransferases are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo- $\alpha_1$  AGP (at 1-10 mg/ml) to compare the ability of the 15 sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo- $\alpha_1$  AGP for this evaluation. Sialyltransferases with the ability to sialylate 20 N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation (as illustrated for ST3Gal III in this disclosure).

*d) Other glycosyltransferases*

One of skill in the art will understand that other glycosyltransferases can be 25 substituted into similar transferase cycles as have been described in detail for the sialyltransferase. In particular, the glycosyltransferase can also be, for instance, glucosyltransferases, *e.g.*, Alg8 (Stagljar *et al.*, *Proc. Natl. Acad. Sci. USA* **91**: 5977 (1994)) or Alg5 (Heesen *et al.*, *Eur. J. Biochem.* **224**: 71 (1994)).

N-acetylgalactosaminyltransferases are also of use in practicing the present 30 invention. Suitable N-acetylgalactosaminyltransferases include, but are not limited to,  $\alpha(1,3)$  N-acetylgalactosaminyltransferase,  $\beta(1,4)$  N-acetylgalactosaminyltransferases (Nagata *et al.*, *J. Biol. Chem.* **267**: 12082-12089 (1992) and Smith *et al.*, *J. Biol. Chem.* **269**: 15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al.*, *J. Biol. Chem.* **268**: 12609

(1993)). Suitable N-acetylglucosaminyltransferases include GnTI (2.4.1.101, Hull *et al.*, *BBRC* **176**: 608 (1991)), GnTII, GnTIII (Ihara *et al.*, *J. Biochem.* **113**: 692 (1993)), GnTIV, and GnTV (Shoreiban *et al.*, *J. Biol. Chem.* **268**: 15381 (1993)), O-linked N-acetylglucosaminyltransferase (Bierhuizen *et al.*, *Proc. Natl. Acad. Sci. USA* **89**: 9326 (1992)), N-acetylglucosamine-1-phosphate transferase (Rajput *et al.*, *Biochem J.* **285**: 985 (1992)), and hyaluronan synthase.

5 Mannosyltransferases are of use to transfer modified mannose moieties.

Suitable mannosyltransferases include  $\alpha(1,2)$  mannosyltransferase,  $\alpha(1,3)$  mannosyltransferase,  $\alpha(1,6)$  mannosyltransferase,  $\beta(1,4)$  mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1 (see, Kornfeld *et al.*, *Annu. Rev. Biochem.* **54**: 631-664 (1985)).

10 Xylosyltransferases are also useful in the present invention. See, for example, Rodgers, *et al.*, *Biochem. J.*, **288**:817-822 (1992); and Elbain, *et al.*, U.S. Patent No., 6,168,937.

15 Other suitable glycosyltransferase cycles are described in Ichikawa *et al.*, *JACS* **114**: 9283 (1992), Wong *et al.*, *J. Org. Chem.* **57**: 4343 (1992), and Ichikawa *et al.* in CARBOHYDRATES AND CARBOHYDRATE POLYMERS. Yaltami, ed. (ATL Press, 1993).

20 Prokaryotic glycosyltransferases are also useful in practicing the invention. Such glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many gram negative bacteria. The LOS typically have terminal glycan sequences that mimic glycoconjugates found on the surface of human epithelial cells or in host secretions (Preston *et al.*, *Critical Reviews in Microbiology* **23**(3): 139-180 (1996)). Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which include a  $\beta$ 1,6 galactosyltransferase and a  $\beta$ 1,3 galactosyltransferase (see, e.g., EMBL Accession Nos. M80599 and M86935 (*E. coli*); EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot Accession No. P25740 (*E. coli*), an  $\beta$ 1,2-glucosyltransferase (*rfaJ*)(Swiss-Prot Accession No. P27129 (*E. coli*)) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an  $\beta$ 1,2-N-acetylglucosaminyltransferase (*rfaK*)(EMBL Accession No. U00039 (*E. coli*)). Other glycosyltransferases for which amino acid sequences are known include those that are encoded by operons such as *rfaB*, which have been characterized in organisms such as *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhimurium*, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprae*, and the *rh1* operon of *Pseudomonas aeruginosa*.

Also suitable for use in the present invention are glycosyltransferases that are involved in producing structures containing lacto-N-neotetraose, D-galactosyl- $\beta$ -1,4-N-acetyl-D-glucosaminyl- $\beta$ -1,3-D-galactosyl- $\beta$ -1,4-D-glucose, and the P<sup>k</sup> blood group trisaccharide sequence, D-galactosyl- $\alpha$ -1,4-D-galactosyl- $\beta$ -1,4-D-glucose, which have been identified in the LOS of the mucosal pathogens *Neisseria gonorrhoeae* and *N. meningitidis* (Scholten *et al.*, *J. Med. Microbiol.* **41**: 236-243 (1994)). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings *et al.*, *Mol. Microbiol.* **18**: 729-740 (1995)) and the *N. gonorrhoeae* mutant F62 (Gotshlich, *J. Exp. Med.* **180**: 2181-2190 (1994)). In *N. meningitidis*, a locus consisting of three genes, *lgtA*, *lgtB* and *lgtE*, encodes the glycosyltransferase enzymes required for addition of the last three of the sugars in the lacto-N-neotetraose chain (Wakarchuk *et al.*, *J. Biol. Chem.* **271**: 19166-73 (1996)). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk *et al.*, *J. Biol. Chem.* **271**(45): 28271-276 (1996)). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds  $\beta$ -D-GalNAc to the 3 position of the terminal galactose of the lacto-N-neotetraose structure and *lgtC* which adds a terminal  $\alpha$ -D-Gal to the lactose element of a truncated LOS, thus creating the P<sup>k</sup> blood group antigen structure (Gotshlich (1994), *supra*). In *N. meningitidis*, a separate immunotype L1 also expresses the P<sup>k</sup> blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.*, (1995), *supra*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553 (Gotschlich). Genes for  $\alpha$ 1,2-fucosyltransferase and  $\alpha$ 1,3-fucosyltransferase from *Helicobacter pylori* has also been characterized (Martin *et al.*, *J. Biol. Chem.* **272**: 21349-21356 (1997)). Also of use in the present invention are the glycosyltransferases of *Campylobacter jejuni* (see, for example, [http://afmb.cnrs-mrs.fr/~pedro/CAZY/gtf\\_42.html](http://afmb.cnrs-mrs.fr/~pedro/CAZY/gtf_42.html)).

## 2. Sulfotransferases

The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al.*, *J. Biol. Chem.* **270**: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al.*, *Genomics* **26**: 239-241

(1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al.*, *J. Biol. Chem.* **269**: 2270-2276 (1994) and Eriksson *et al.*, *J. Biol. Chem.* **269**: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

5

### 3. *Cell-Bound Glycosyltransferases*

In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen *et al.*, *Proc. Natl. Acad. Sci. USA* **86**: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:β-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

Francisco *et al.*, *Proc. Natl. Acad. Sci. USA* **89**: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to prokaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

#### **4. Fusion Proteins**

In other exemplary embodiments, the methods of the invention utilize fusion proteins that have more than one enzymatic activity that is involved in synthesis of a desired glycopeptide conjugate. The fusion polypeptides can be composed of, for example, a 5 catalytically active domain of a glycosyltransferase that is joined to a catalytically active domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar that is a donor for the glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle. For example, a polynucleotide that encodes a glycosyltransferase can be joined, in-frame, to a 10 polynucleotide that encodes an enzyme involved in nucleotide sugar synthesis. The resulting fusion protein can then catalyze not only the synthesis of the nucleotide sugar, but also the transfer of the sugar moiety to the acceptor molecule. The fusion protein can be two or more cycle enzymes linked into one expressible nucleotide sequence. In other embodiments the fusion protein includes the catalytically active domains of two or more glycosyltransferases.

15 See, for example, 5,641,668. The modified glycopeptides of the present invention can be readily designed and manufactured utilizing various suitable fusion proteins (see, for example, PCT Patent Application PCT/CA98/01180, which was published as WO 99/31224 on June 24, 1999.)

#### **20 5. Immobilized Enzymes**

In addition to cell-bound enzymes, the present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate 25 is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

30

#### **Purification of FSH Conjugates**

The products produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of glycosylated saccharides such as thin or thick layer

chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins such as glycosyl transferases. Nanofiltration or reverse osmosis can then be used to remove salts and/or purify the product saccharides (see, e.g., WO 98/15581). Nanofilter membranes are a class of reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to 10 about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, saccharides prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

If the modified glycoprotein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by 15 centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (e.g., on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-20 Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (e.g., silica gel with appended aliphatic groups), gel filtration using, e.g., Sephadex molecular sieve or size-exclusion chromatography, 25 chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

Modified glycopeptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the modified 30 glycoprotein may be purified by affinity chromatography. Finally, HPLC may be employed for final purification steps.

A protease inhibitor, e.g., methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

Within another embodiment, supernatants from systems which produce the modified glycopeptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification 5 matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Alternatively, a cation-exchange step may be employed. Suitable 10 cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a polypeptide variant composition. Some or all of the foregoing purification 15 steps, in various combinations, can also be employed to provide a homogeneous modified glycoprotein.

The modified glycopeptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al.*, *J. Chromatog.* 296: 171 (1984). This reference describes two sequential, RP-HPLC steps for 20 purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

### **Pharmaceutical Compositions**

In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable diluent and a covalent 25 conjugate between a non-naturally-occurring, PEG moiety, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group 30 interposed between and covalently linked to both the peptide and the polymer, therapeutic moiety or biomolecule.

Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th

ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* 249:1527-1533 (1990).

The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, 5 intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable 10 microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Commonly, the pharmaceutical compositions are administered parenterally, e.g., intravenously. Thus, the invention provides compositions for parenteral administration 15 which comprise the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, e.g., water, buffered water, saline, PBS and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

20 These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

25 In some embodiments the glycopeptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (e.g., the sialyl galactosides of the invention) is 30 well known in the art (*see*, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044).

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized glycopeptides of the invention.

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (e.g., alkylation or acylation of a hydroxyl group present on the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively). Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the aqueous surface of the liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface.

The compounds prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with  $^{125}\text{I}$ ,  $^{14}\text{C}$ , or tritium.

The active ingredient used in the pharmaceutical compositions of the present invention is glycopegylated FSH. The liposomal dispersion of the present invention is useful as a parenteral formulation in treating infertility. Preferably, the FSH composition of the present invention is administered parenterally (e.g. IV, IM, SC or IP). Effective dosages are expected to vary considerably depending on the condition being treated and the route of administration but are expected to be in the range of about 0.1 (~7U) to 100 (~7000U)  $\mu\text{g}/\text{kg}$  body weight of the active material. Preferable doses for treatment of anemic conditions are about 50 to about 300 Units/kg three times a week. Because the present invention provides a FSH with an enhanced in vivo residence time, the stated dosages are optionally lowered when a composition of the invention is administered.

The following examples are provided to illustrate the conjugates, and methods and of the present invention, but not to limit the claimed invention.

## EXAMPLES

Example 1 illustrates the assembly of a conjugate of the invention. Follicle Stimulating Hormone (FSH) is desialylated. The desialylated FSH is conjugated with CMP-(sialic acid)-PEG.

5

### EXAMPLE 1

This example illustrates the assembly of a conjugate of the invention. Follicle Stimulating Hormone (FSH) is desialylated and then conjugated with CMP-(sialic acid)-PEG.

#### Desialylation of Follicle Stimulating Hormone

Follicle Stimulating Hormone (FSH) (Human Pituitary, Calbiochem Cat No.

10 869001), 1 mg, was dissolved in 500  $\mu$ L 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>. This solution, 375  $\mu$ L, was transferred to a small plastic tube and to it was added 263 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was shaken gently for 15 hours at 32 °C. The reaction mixture was added to N-(*p*-aminophenyl)oxamic acid-agarose conjugate, 600  $\mu$ L, pre-equilibrated with 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% 15 NaN<sub>3</sub> and gently rotated 6.5 hours at 4 °C. The suspension was centrifuged for 2 minutes at 14,000 rpm and the supernatant was collected. The beads were washed 5 times with 0.5 mL of the buffer and all supernatants were pooled. The enzyme solution was dialyzed (7000 MWCO) for 15 hours at 4 °C with 2 L of a solution containing 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>, and then twice for 4 hours at 4 °C into 50 mM Tris -HCl pH 7.4, 1 M 20 NaCl, 0.05% NaN<sub>3</sub>. The solution was concentrated to 2  $\mu$ g/ $\mu$ L by Speed Vac and stored at –20 °C. Reaction samples were analyzed by IEF gels (pH 3-7) (Invitrogen).

#### Preparation of human pituitary-derived SA-FSH and PEG-SA-Follicle Stimulating Hormone

Desialylated FSH (100  $\mu$ g, 50  $\mu$ L) and CMP-sialic acid or CMP-SA-PEG (1

25 kDa or 10 kDa) (0.05  $\mu$ mol) were dissolved in 13.5  $\mu$ L H<sub>2</sub>O (adjusted to pH 8 with NaOH) in 0.5 mL plastic tubes. The tubes were vortexed briefly and 40 mU ST3Gal3 (36.5  $\mu$ L) was added (total volume 100  $\mu$ L). The tubes were vortexed again and shaken gently for 24 hours at 32 °C. The reactions were stopped by freezing at –80 °C. Reaction samples of 15  $\mu$ g were analyzed by SDS-PAGE, IEF gels and MALDI-TOF. Native FSH was also analyzed by 30 SDS-PAGE.

### **Analysis of SDS PAGE and IEF Gels of Reaction Products**

Novex Tris-Glycine 8-16% 1 mm gels for SDS PAGE analysis were purchased from Invitrogen. 7.5  $\mu$ L (15  $\mu$ g) of FSH reaction samples were diluted with 5  $\mu$ L of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer, mixed with 15  $\mu$ L sample 5 loading buffer and 1  $\mu$ L 9 M  $\mu$ -mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen and stained with Colloidal Blue Stain (Invitrogen).

FSH samples (15  $\mu$ g) were diluted with 5  $\mu$ L Tris buffer and mixed with 15  $\mu$ L sample loading buffer. The samples were then applied to Isoelectric Focusing Gels (pH 3-7) (Invitrogen). Gels were run and fixed as directed by Invitrogen and then stained with 10 Colloidal Blue Stain.

### **EXAMPLE 2**

#### **GlycoPEGylation of recombinant FSH produced recombinantly in CHO cells**

This example illustrates the assembly of a conjugate of the invention.

Desialylated FSH was conjugated with CMP-(sialic acid)-PEG.

#### **15 Preparation of recombinant Asialo-Follicle Stimulating Hormone**

Recombinant Follicle Stimulation Hormone (rFSH) produced from CHO was used in these studies. The 7,500 IU of rFSH was dissolved in 8 mL of water. The FSH solution was dialyzed in 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500  $\mu$ L in a Centricon Plus 20 centrifugal filter. A portion of this solution 20 (400  $\mu$ L) (~ 0.8 mg FSH) was transferred to a small plastic tube and to it was added 275 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was mixed for 16 hours at 32 °C. The reaction mixture was added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800  $\mu$ L) and gently rotated for 24 hours at 4 °C. The mixture was centrifuged at 10,000 rpm and the supernatant was collected. The beads were washed 3 times with 0.6 mL 25 Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants were pooled. The supernatant was dialyzed at 4 °C against 2 L of 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution was then concentrated to 420  $\mu$ L in a Centricon Plus 20 centrifugal filter and stored at -20 °C.

30 Native and desialylated rFSH samples were analyzed by SDS-PAGE and IEF. Novex Tris-Glycine 8-16% 1 mm gels were purchased from Invitrogen. Samples (7.5  $\mu$ L, 15  $\mu$ g) samples were diluted with 5  $\mu$ L of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05%

NaN<sub>3</sub> buffer, mixed with 15  $\mu$ L sample loading buffer and 1  $\mu$ L 9 M  $\beta$ -mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen and stained with Colloidal Blue Stain (Invitrogen). Isoelectric Focusing Gels (pH 3-7) were purchased from Invitrogen. Samples (7.5  $\mu$ L, 15  $\mu$ g) were diluted with 5  $\mu$ L Tris buffer and mixed with 15 5  $\mu$ L sample loading buffer. Gels were loaded, run and fixed as directed by Invitrogen. Gels were stained with Colloidal Blue Stain. Samples of native and desialylated FSH were also dialyzed against water and analyzed by MALDI-TOF.

#### **Sialyl-PEGylation of recombinant Follicle Stimulation Hormone**

Desialylated FSH (100  $\mu$ g, 54  $\mu$ L) and CMP-SA-PEG (1 kDa or 10 kDa) (0.05 10  $\mu$ mol) were dissolved in 28  $\mu$ L 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2 in 0.5 mL plastic tubes. The tubes were vortexed briefly and 20 mU of ST3Gal3 was added (total volume 100  $\mu$ L). The tubes were vortexed again, mixed gently for 24 hours at 32 °C and the reactions stopped by freezing at -80 °C. Samples of this reaction were analyzed as described above by SDS-PAGE gels, IEF gels and MALDI-TOF MS.

15 MALDI was also performed on the PEGylated rFSH. During ionization, SA-PEG is eliminated from the N-glycan structure of the glycoprotein. Native FSH gave a peak at 13928; AS-rFSH (13282); resialylated r-FSH (13332); PEG1000-rFSH (13515; 14960 (1); 16455 (2); 17796 (3); 19321 (4)); and PEG 10000 (23560 (1); 34790 (2); 45670 (3); and 56760 (4)).

#### **EXAMPLE 3**

##### **Pharmacokinetic Study of GlycoPEGylated FSH**

This example sets forth the *in vivo* testing of the pharmacokinetic properties of glycoPEGylated Follicle Stimulating Hormone (FSH) prepared according to the methods of the invention as compared to non-PEGylated FSH.

25 FSH, FSH-SA-PEG (1 kDa) and FSH-SA-PEG (10 kDa) were radioiodinated using standard conditions (Amersham Biosciences, Arlington Heights, IL) and formulated in phosphate buffered saline containing 0.1% BSA. After dilution in phosphate buffer to the appropriate concentration, each of the test FSH proteins (0.4  $\mu$ g, each) was injected intraveneously into female Sprague Dawley rats (250-300 g body weight) and blood drawn at 30 time points from 0 to 80 hours. Radioactivity in blood samples was analyzed using a gamma counter and the pharmacokinetics analyzed using standard methods. FSH was cleared from the blood much more quickly than FSH-PEG(1 kDa), which in turn was clear somewhat more quickly than FSH-PEG(10 kDa).

#### EXAMPLE 4

##### **Sertoli Cell Bioassay for *In Vitro* Activity of GlycoPEGylated FSH**

This example sets forth a bioassay for follicle stimulating hormone (FSH) activity based on cultured Sertoli cells. This assay is useful to determine the bioactivity of FSH after glycan remodeling, including glycoconjugation.

This bioassay is based on the dose-response relationship that exists between the amount of estradiol produced when FSH, but not lutenizing hormone (LH), is added to cultured Sertoli cells obtained from immature old rats. Exogenous testosterone is converted to 17 $\beta$ -estradiol in the presence of FSH.

10            Seven to 10 days old Sprague-Dawley rats were used to obtain Sertoli cells. After sacrifice, testes were decapsulated and tissue was dispersed by incubation in collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml) for 5 to 10 min. The tubule fragments settled to the bottom of the flask and were washed in PBS (1x). The tubule fragments were reincubated for 20 min with a media containing the same 15 enzymes: collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml).

The tubule fragments were homogenized and plated into a 24 well plate in a serum free media.  $5 \times 10^5$  cells were dispersed per well. After 48h incubation at 37° C and 5% CO<sub>2</sub>, fresh media was added to the cells. Composition of the serum free media: DMEM 20 (1 vol), Ham's F10 nutrient mixture (1 vol), insulin 1  $\mu$ g/ml, Transferrin 5  $\mu$ g/ml, EGF 10 ng/ml, T4 20 pg/ml, Hydrocortisone 10<sup>-8</sup> M, Retinoic acid 10<sup>-6</sup> M.

The stimulation experiment consists of a 24 hour incubation with standard FSH or samples at 37° C and 5% CO<sub>2</sub>. The mean intra-assay coefficient of variation is 9% and the mean inter-assay coefficient of variation is 11%.

25            The 17B-estradiol Elisa Kit DE2000 (R&D Systems, Minneapolis, MN) was used to quantify the level of estradiol after incubation with FSH, FSH-SA-PEG (1 kDa) and FSH-SA-PEG (10 kDa).

The procedure was as follows: 100  $\mu$ l of Estradiol Standard (provided with kit and prepared as per instructions with kit) or sample was pipetted into wells of 17B-estradiol 30 Elisa plate(s); 50  $\mu$ l of 17B-estradiol Conjugate (provided with kit, prepared as per instructions with kit) was added to each well; 50  $\mu$ l of 17B-estradiol antibody solution (provided with kit and prepared as per instructions with kit) was added to each well; plates were incubated for 2 hour at room temperature at 200 rpm; the liquid was aspirated from each

well; the wells were washed 4 times using the washing solution; all the liquid was removed from the wells; 200  $\mu$ L of pNPP Substrate (provided with kit and prepared as per instructions with kit) was added to all wells and incubated for 45 min; 50  $\mu$ L of Stop solution (provided with kit and prepared as per instructions with kit) was added and the plates were read it at 405 nm. While FSH-PEG(10 kDa) exhibited a modest stimulation of Sertoli cells, at 1  $\mu$ g/ml, FSH-PEG(1 kDa) stimulated Sertoli cells up to 50% more than unPEGylated FSH.

## EXAMPLE 5

### Steelman-Pohley Bioassay of *In Vivo* Activity of GlycoPEGylated FSH

In this example, the Steelman-Pohley bioassay (Steelman and Pohley, 1953, Endocrinology 53:604-615) was used to determine the *in vivo* activity of glycoPEGylated FSH. The Steelman-Pohley assay uses the change in ovary weight of a rat to measure the *in vivo* activity of FSH that is coinjected with human chorionic gonadotropin.

The Steelman-Pohley bioassay was performed according to the protocol described in Christin-Maitre et al. (2000, *Methods* 21:51-57). Seventy female Sprague-Dawley Rats (Charles River Laboratories, Wilmington, MA), aged 21 to 22 days, were housed in the testing facility for at least 5 days before the beginning the assay procedure. Throughout the procedure, the animal room was climate controlled at 18 to 26°C, 30 to 70% relative humidity, and 12 hr. artificial light/12 hr. dark. All animals were fed Certified Rodent Chow (Harlan Teklad, Madison WI) or the equivalent, and water, both *ad libitum*. Animal procedures were performed at Calvert Preclinical Services, Inc. (Olyphant, PA).

Recombinant FSH was expressed in CHO cells, purified by standard techniques and glycoPEGylated with PEG (1 kDa). The rats were divided into seven test groups, with ten animals per group. On days -1 and 0, animals of all groups were subcutaneously injected with 20 I.U. of human chorionic gonadotropin (HCG) in 0.5 ml of 0.9 % NaCl. On days 1, 2 and 3, the control animals were subcutaneously injected with a dose of 0.5 ml containing 20 I.U. HCG in 0.9% NaCl, while in the other groups, the HCG dose was augmented with either rFSH or rFSH-SA-PEG (1 kDa) at either 0.14  $\mu$ g, 0.4  $\mu$ g or 1.2  $\mu$ g per dose. On day 4, the animals were euthanized by CO<sub>2</sub> inhalation. The ovaries were removed, trimmed and weighted. The average ovary weight was determined for each group.

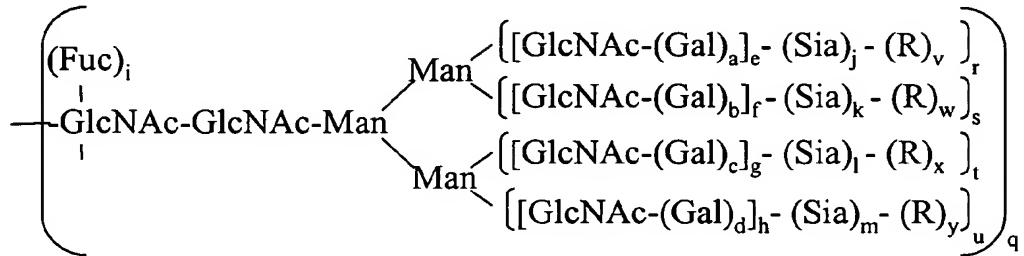
The groups receiving HCG alone (control) or the low dose (0.14  $\mu$ g) of either rFSH or rFSH-SA-PEG (1 kDa) had ovary weights that were roughly equivalent. The groups receiving the medium (0.4  $\mu$ g) or high (1.2  $\mu$ g) doses of rFSH or rFSH-SA-PEG (1 kDa) had ovary weights roughly twice that of the control group. At the medium dose (0.4  $\mu$ g), the

glycoPEGylated rFSH had roughly the same *in vivo* activity (as determined by ovary weight) as the unPEGylated rFSH. At the high dose (1.2  $\mu$ g), the glycoPEGylated rFSH had somewhat higher *in vivo* activity than the unPEGylated rFSH.

It is understood that the examples and embodiments described herein are for 5 illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

**WHAT IS CLAIMED IS:**

1                   1.     A follicle stimulating polypeptide comprising a glycosyl residue  
2     having the formula:



wherein

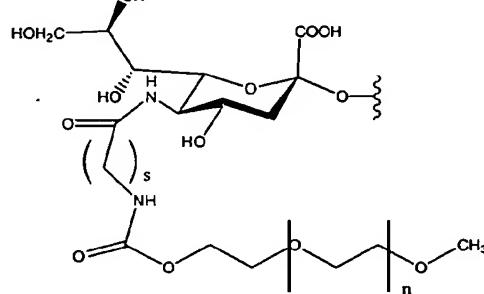
a, b, c, d, i, q, r, s, t, and u are members independently selected from 0 and 1;

e, f, g, and h are members independently selected from the integers between 0 and 6;

j, k, l, and m are members independently selected from the integers between 0 and 100;

v, w, x, and y are 0; and  
Sia-(R) has the formula:

Sta (1) has the formula:



in which  $n$  is an integer from 0 to 500.

1 2. The conjugate according to claim 1, wherein said peptide has the  
2 sequence:

3 APDVQDCPECTLQENPFFSQPGAPILCQCMGCCFSRAYPTPLRSKKTMLVQKNVTS  
4 ESTCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKSNSCELTNITIAIEKEECRFC  
5 ISINTTWCAGCYTRDLVYKDPARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYP  
6 VATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE.

1                   4.        A pharmaceutical formulation comprising the peptide according to  
2    claim 1 and a pharmaceutically acceptable carrier.

1                   5.        A method of stimulating ovarian follicles in a mammal, said method  
2    comprising administering to said mammal a peptide according to claim 1.

1                   6.        A method of treating reproductive infertility in a subject, said method  
2    comprising administering to said subject a peptide according to claim 1.

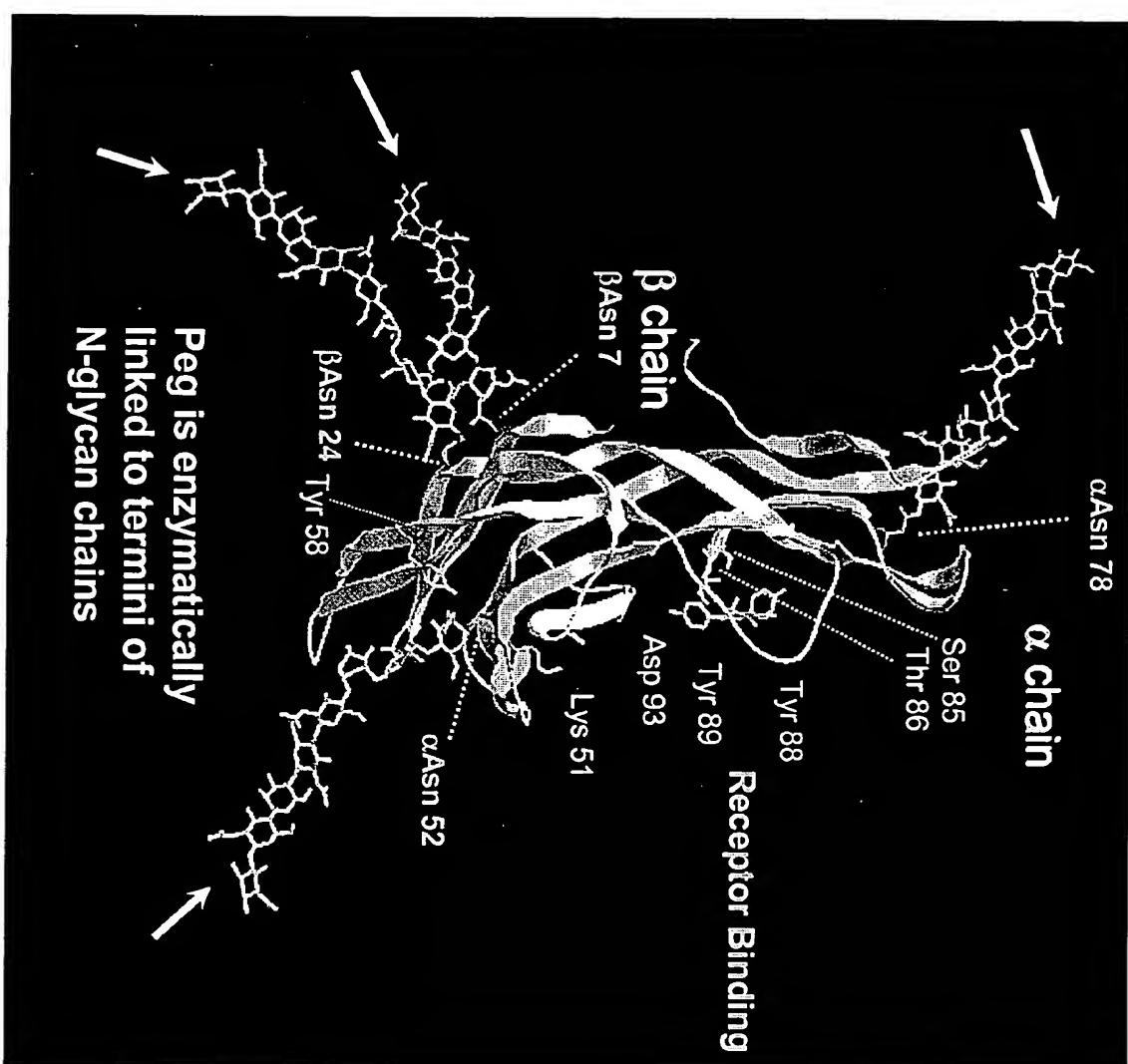
## **GLYCOPEGYLATED FOLLICLE STIMULATING HORMONE**

### **ABSTRACT OF THE DISCLOSURE**

The present invention provides conjugates between follicle stimulating hormone and PEG moieties. The conjugates are linked via an intact glycosyl linking group that is interposed between and covalently attached to the peptide and the modifying group. The conjugates are formed from both glycosylated and unglycosylated peptides by the action of a glycosyltransferase. The glycosyltransferase ligates a modified sugar moiety onto either an amino acid or glycosyl residue on the peptide. Also provided are pharmaceutical formulations including the conjugates. Methods for preparing the conjugates are also within the scope of the invention.

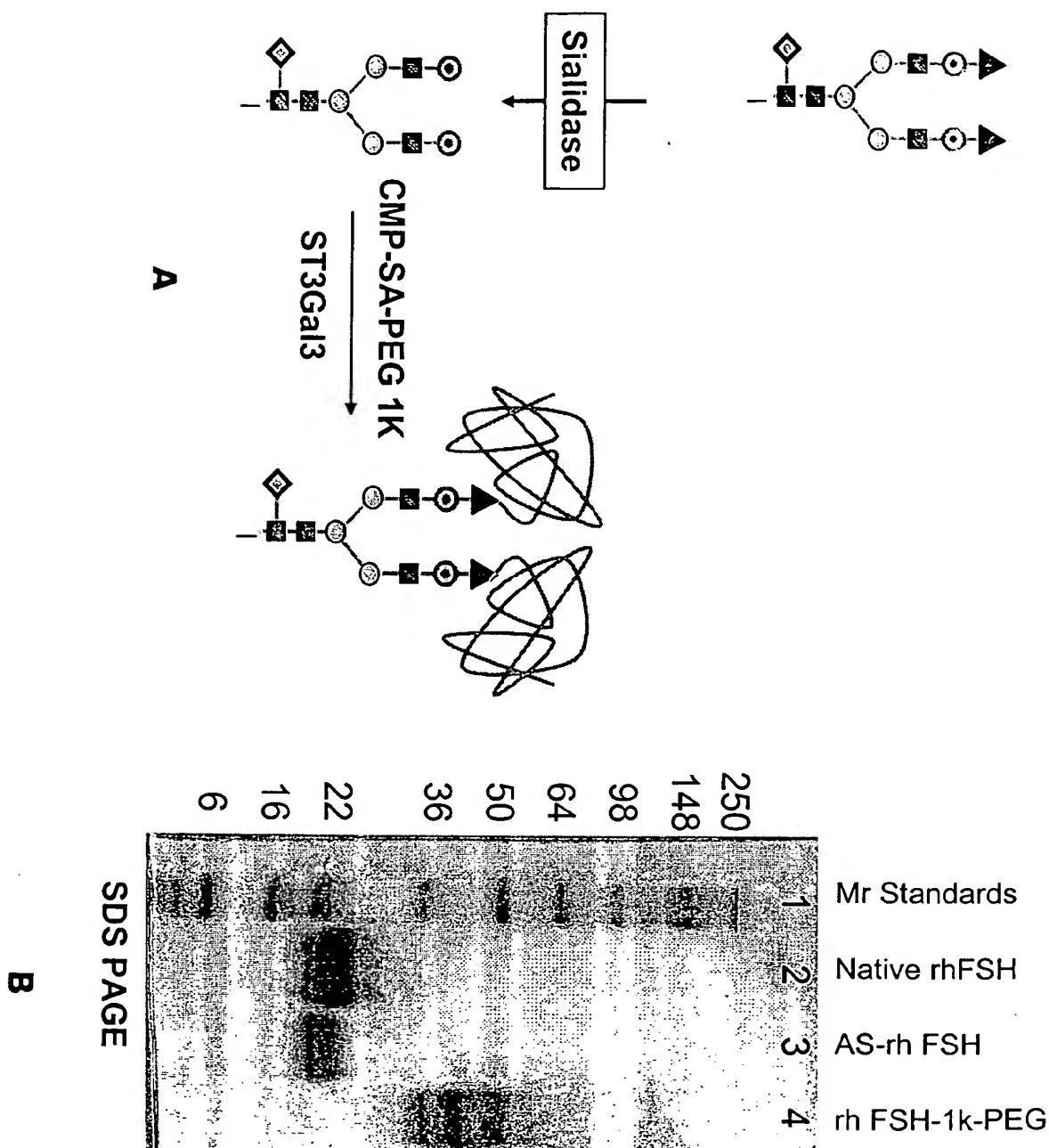
60093606 v1

FIG. 1



BEST AVAILABLE COPY

FIG. 2



BEST AVAILABLE COPY

FIG. 3

Rat i.v. bolus injection PK model

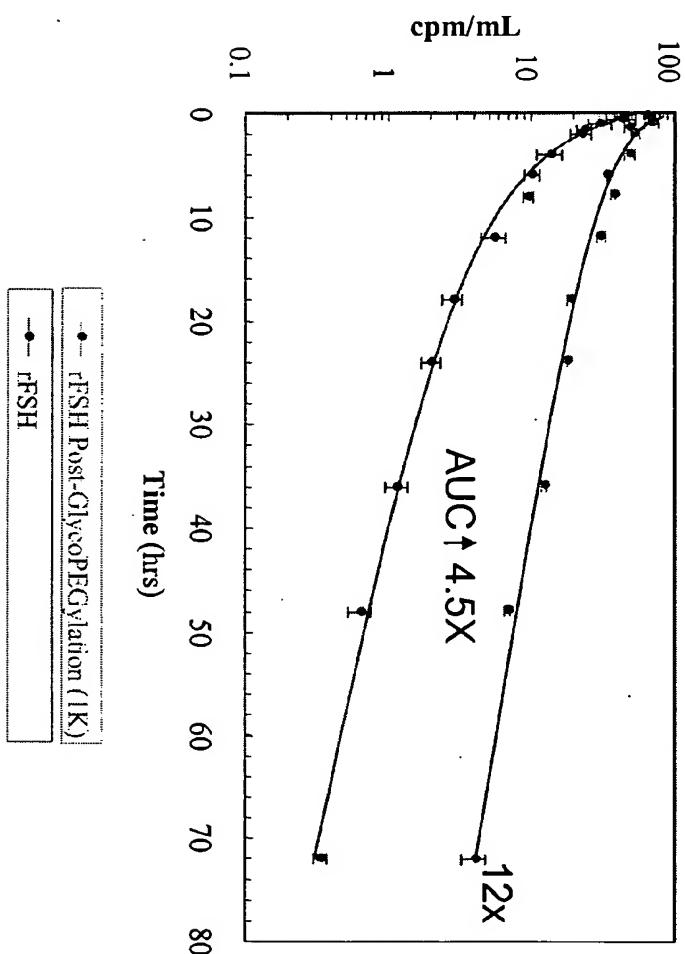
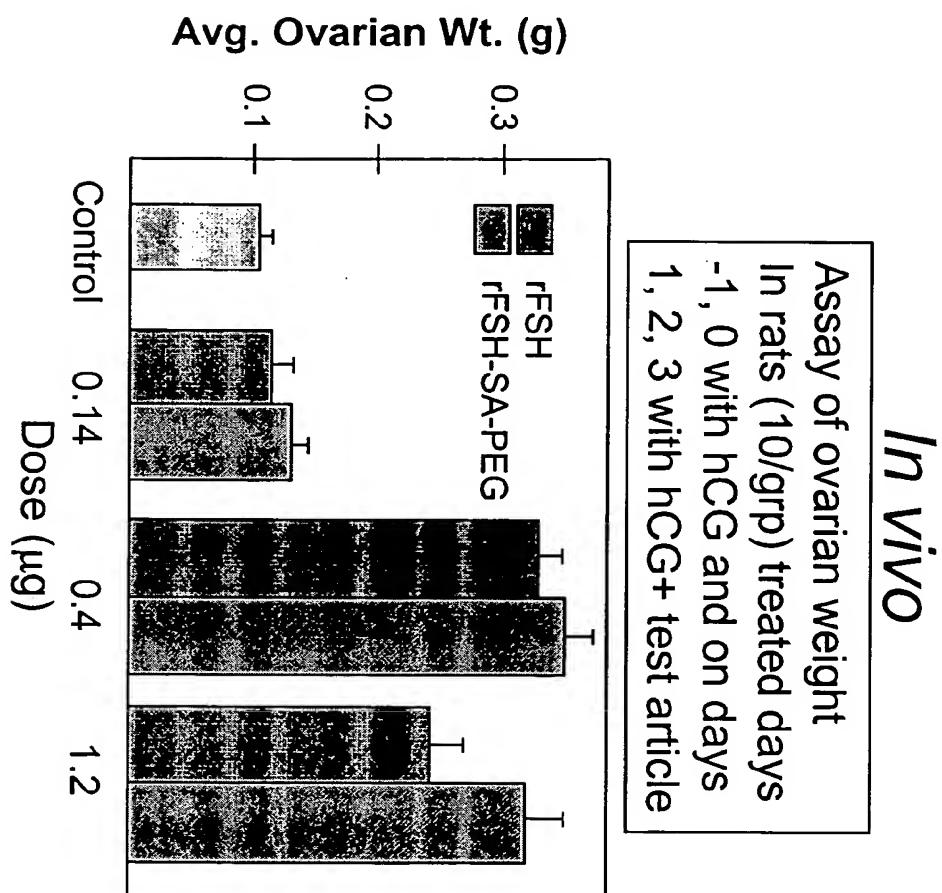
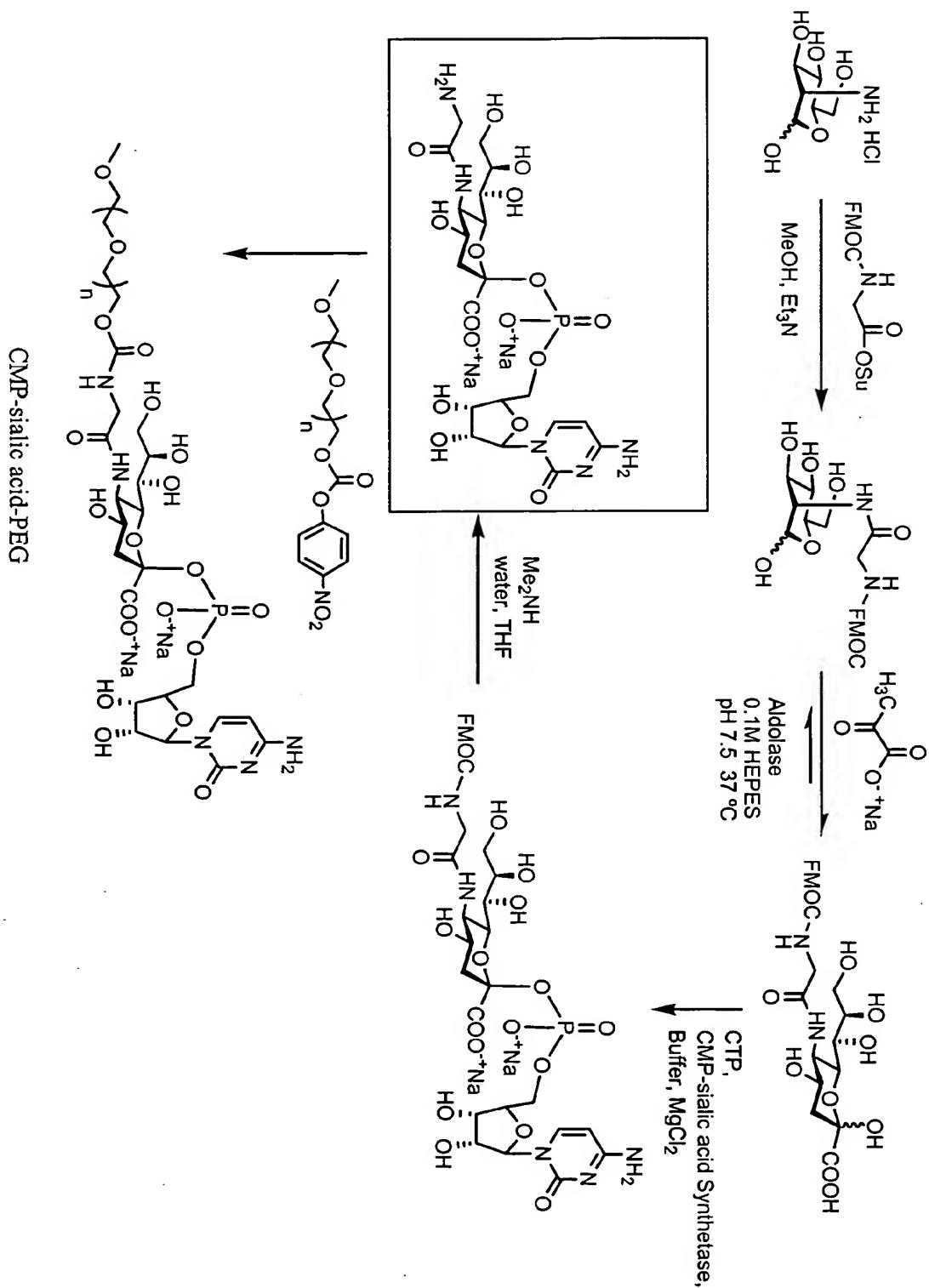


FIG. 4



BEST AVAILABLE COPY

FIG. 5



**FIG. 6**

APDVQDCPECTLQENPFFSQPGAPILCQCMGCCFSRAYPTPLRSKKTMLVQKNVTSE  
STCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKSNSCELTNITIAIEKEECRFCISI  
NTTWCAGCYTRDLVYKDPARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVAT  
QCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE

## Application Data Sheet

## Application Information

Application number::

Filing Date::

Application Type:: **Provisional**

Subject Matter:: Utility

#### Suggested classification::

### Suggested Group Art

## CD-ROM or CD-R??::

Number of CD disks::

Number of copies of CDs::

### Sequence Submission::

### Computer Readable Form (CRF)?::

Number of copies of CRF::

**Title:: GLYCOPEGYLATED HUMAN FOLLICLE STIMULATING HORMONE**

Attorney Docket Number:: 19957-019300US

**Request for Early Publication::** No

**Request for Non-Publication::** No

### Suggested Drawing Figure::

Total Drawing Sheets:: 6

Small Entity?:: Yes

Latin name::

Variety denomination name: -

Petition included?:: No

Petition Type::

Licensed US Govt. Agency::

**Contract or Grant Numbers One::**

Secrecy Order in Parent Appl.: No

## **Applicant Information**

Applicant Authority Type:: Inventor  
Primary Citizenship Country:: US  
Status:: Full Capacity  
Given Name:: Shawn  
Middle Name::  
Family Name:: DeFrees  
Name Suffix::  
City of Residence:: North Wales  
State or Province of Residence:: PA  
Country of Residence:: US  
Street of Mailing Address:: 126 Filly Drive  
City of Mailing Address:: North Wales  
State or Province of mailing address:: PA  
Country of mailing address:: US  
Postal or Zip Code of mailing address:: 19454

Applicant Authority Type:: Inventor  
Primary Citizenship Country:: US  
Status:: Full Capacity  
Given Name:: Robert  
Middle Name:: J.  
Family Name:: Bayer  
Name Suffix::  
City of Residence:: San Diego  
State or Province of Residence:: CA  
Country of Residence:: US  
Street of Mailing Address:: 6105 Dirac Street  
City of Mailing Address:: San Diego  
State or Province of mailing address:: CA

Country of mailing address:: US  
Postal or Zip Code of mailing address:: 92122

**Correspondence Information**

Correspondence Customer Number:: 20350

**Representative Information**

Representative Customer Number:: 20350

**Domestic Priority Information**

Application:: Continuity Type:: Parent Application:: Parent Filing Date::

**Foreign Priority Information**

Country:: Application number:: Filing Date::

**Assignee Information**

Assignee Name::

Street of mailing address::

City of mailing address::

State or Province of mailing address::

Country of mailing address::

Postal or Zip Code of mailing address::